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Improved use of abattoir information to aid the management of liver fluke in cattle

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Submitted for the degree of Doctor of Philosophy

THE UNIVERSITY
of EDINBURGH

2016

Dedication

Στην οικογένειά μου: σ΄ αυτούς που ήταν και σ΄ αυτούς που έγιναν.

Declaration

This thesis is submitted to the University of Edinburgh in accordance with the requirements for the degree of Doctor of Philosophy in the faculty of Medicine and Veterinary Medicine. The work presented is the work of the author except where stated otherwise by reference and/or acknowledgement. Any work presented, which has been conducted by (or in collaboration with) others is explicitly acknowledged. No part of this work has been submitted for award or degree at any other university.

Signed:

Stella Mazeri

Thesis abstract

Fasciolosis, caused by the trematode parasite *Fasciola hepatica*, is a multi-host parasitic disease affecting many countries worldwide. It is a well-recognized clinically and economically important disease of food producing animals such as cattle and sheep. In the UK, the incidence and distribution of fasciolosis has been increasing in the last decade while the timing of acute disease is becoming more variable and the season suitable for parasite development outside the mammalian host has been extended. Meanwhile control is proving increasingly difficult due to changing weather conditions, increased animal movements and developing anthelmintic resistance.

Forecasting models have been around for a long time to aid health planning related to fasciolosis control, but studies identifying management related risk factors are limited. Moreover, the lack of information on the accuracy of meat inspection and available liver fluke diagnostic tests hinders effective monitoring of disease prevalence and treatment. So far, the evaluation of tests available for the diagnosis of the infection in cattle has mainly been carried out using gold standard approaches or under experimental settings, the limitations of which are well known. In cattle, the infection mainly manifests as a sub-clinical disease, resulting in indirect production losses, which are difficult to estimate. The lack of obvious clinical signs results in these losses commonly being attributed to other causes such as poor weather conditions or bad quality forage. This further undermines establishment of appropriate control strategies, as it is difficult to convince farmers to treat without demonstrating clear economic losses of sub-clinical disease.

This project explores the value of slaughterhouse data in understanding the changing epidemiology of fasciolosis, identifying sustainable control measures and estimating the effect of infection on production parameters using data collected at one of the

largest cattle and sheep abattoirs in Scotland. Data used in this study include; a) abattoir data routinely collected during 2013 and 2014, b) data collected during 3 periods of abattoir based sampling, c) data collected through administration of a management questionnaire and d) climatic and environmental data from various online sources.

A Bayesian extension of the Hui Walter no gold standard model was used to estimate the diagnostic sensitivity and specificity of five diagnostic tests for fasciolosis in cattle, which were applied on 619 samples collected from the abattoir during three sampling periods; summer 2013, winter 2014 and autumn 2014. The results provided novel information on the performance of these tests in a naturally infected cattle population at different times of the year. Meat inspection was estimated to have a sensitivity of 0.68 (95% BCI 0.61-0.75) and a specificity of 0.88 (95% BCI 0.85-0.91). Accurate estimates of sensitivity and specificity will allow for routine abattoir liver inspection to be used as a tool for monitoring the epidemiology of *F. hepatica* as well as evaluating herd health planning.

Linear regression modelling was used to estimate the delay in reaching slaughter weight in beef cattle infected with *F. hepatica*, accounting for other important factors such as weight, age, sex, breed and farm as a random effect. The model estimated that cattle classified as having fluke based on routine liver inspection had on average 10 (95% CI 9-12) days greater slaughter age, assuming an average carcass weight of 345 kg. Furthermore, estimates from a second model indicated that the increase in age at slaughter was more severe for higher fibrosis scores. More precisely, the increase in slaughter age was 34 (95% CI 11-57) days for fibrosis score of 1, 93 (95% CI 57-128) days for fibrosis score 2 and 78 (95% CI 30-125) days for fibrosis score 3. Similarly, in a third model comparing different burden categories with animals with no fluke burden, there was a 31 (95% CI 7-56) days increase in slaughter age for animals with 1 to 10

parasites and 77 (95% CI 32-124) days increase in animals with more than 10 parasites found in their livers.

Lastly, a multi-variable mixed effects logistic regression model was built to estimate the association between climate, environmental, management and animal specific factors and the risk of an animal being infected by *F. hepatica*. Multiple imputation methodology was employed to deal with missing data arising from skipped questions in the questionnaire. Results of the regression model confirmed the importance of temperature, rainfall and cattle movements in increasing the risk for fasciolosis, while it indicated that the presence of deer can increase the risk of infection and that male cattle have a reduced risk of infection.

Overall, this project has used slaughterhouse data to fill important knowledge gaps regarding *F. hepatica* infection in cattle. It has provided valuable information on the accuracy of routine abattoir meat inspection, as well as other diagnostic tests. It has also provided estimates of the effect of infection on the time cattle take to reach slaughter weight at different levels of infection and identified relevant risk factors related to the infection. In conclusion, knowledge of the effect of infection on slaughter age, as well as regional risk factors for *F. hepatica* infection, along with an improved use of abattoir inspection results in the evaluation of treatment strategies, can provide farmers and veterinarians with better incentives and tools to improve their herd health strategies and in the longer term help reduce the incidence of liver fluke in cattle.

Acknowledgments

I would like to express my sincere gratitude to everybody who has helped me through this challenging and enjoyable journey. I would like to thank my PhD supervisors Professor Neil Sargison, Dr Ian Handel and Dr Mark Bronsvoort for their invaluable support during the whole time of research and writing of this thesis. I would like to thank Professor Neil Sargison for sharing with me his interest and passion on parasites and specifically liver fluke, inspiring me by example to work long hours in the abattoir and in the lab, to keep coming up with questions and to keep my work scientific yet applicable so that it can have an impact. Neil has helped me greatly in understanding the world of liver fluke within the UK farming system, guided me through Faecal Egg Counting, liver dissections, helped me with the questionnaire design and despite his very busy schedule has found time to join me in the abattoir numerous times during both the design as well as the implementation of the abattoir based sampling.

I have been working with Ian Handel and Mark Bronsvoort since my MSc thesis in 2011 and I was lucky to be able to continue working with them during my PhD. I would like to thank them for encouraging me to pursue a career in veterinary epidemiology. They have been extremely supportive from the very fist day we met and their trust and support encouraged me to work hard, focus on designing epidemiologically sound studies and investigate different statistical methodologies. Ian has offered me valuable help and guidance with the statistical analysis, especially of the first data chapter, the questionnaire design and sampling design. Mark has helped me greatly with the questionnaire design, sampling design and statistical analysis and has joined the abattoir based sampling on several occasions.

An important part of this PhD involved fieldwork and labwork. Danielle Gordon was the first person to introduce me to the world of liver fluke diagnostics, showing me how to perform Faecal Egg Counting and the copro-antigen ELISA. I would like to thank Danielle for hosting me at the Moredun Institute and giving me an introduction to these tests and for later coming over to help me set up the ELISA in our own lab.

I would like to thank everybody at the Department of Veterinary Parasitology at the University of Liverpool for welcoming me to their lab and especially Catherine Hartley and Diana Williams for spending time with me in Liverpool to show me how to perform their in-house serum antibody ELISA, for providing me with positive and negative controls, and for supporting me through email when I was carrying out the ELISA in Edinburgh.

I would like to thank Spiridoula Athanasiadou and late Steve Bishop for their invaluable input, guidance and support as members of my PhD committee.

Sampling days were long and tiring and would have been impossible without all the people who woke up before dawn to come out and help! Thank you so much Adrian Mwonge, Anthi Miliadou, Chloe Davison, Emily Francis, Franklyn Nkongho, Hicham, Kathrin Schaten, Louise Soutar, Mark Bronsvoort, Neil Sargison, Paolo Motta, Rob Kelly, Siben Li, Stuart Dawes and Tina Liu for basically making a large part of this PhD possible.

A special mention goes to Rob Kelly who has been my PhD buddy from day one! Rob has helped me with the questionnaire, setting up the serum antibody ELISA in the lab, setting up and carrying out some of the fieldwork and a lot more. Most importantly, we spent lots of time drinking coffee (Cameroonian, Malawian, Greek and sometimes Dolly's coffee) complaining about our PhDs - as PhD students do -, only to help each other find solutions and get on with it! So thank you so much Rob!

I would like to thank Gustaf for the hours or days we have spent trying to decide which is the best statistical methodology to use for the second data chapter, running Bayesian models for days only to end up using a frequentist approach in the end. Thank you for all the interesting statistical (and not) chats, for being such a patient teacher and for making me realise I have so much more to learn, but it's worth it! I would like to thank Paul for helping me with obtaining and extracting the climate and environmental data for the last data chapter of this PhD and for always keeping the spirits up with one of his clever jokes! I would like to thank Paolo and Siben for being such good office

neighbourghs, tequila drinking partners and colleagues!

As most students would vouch for, a PhD can be tricky at times to say the least and it would be impossible to get over such 'crises' without the support of a great working environment, but most importantly friends and family. Four years is a long time and a lot of people have come and gone, as they normally do in Edinburgh! Adrian, Anna, Anthi, Antonio, Aris, Chara, Corina, Despina, Maria, Martina, Miriam, Sam and Yannis I would like to thank you all for being around these last four years during the fun and the less fun times we've had together. Thank you for being great flatmates, band mates, syn festival buddies, good friends, for helping me at work, for proofreading, for cooking amazing food for me etc. I couldn't have done this without you!

I've been a student for almost 10 years now and possibly the ones to blame the most for inspiring me, encouraging me, supporting me and even pushing me towards this are of course my parents Kostas and Maria, my sister Georgia, my grandmother Stella and everybody else in my family. So thank you very much for enduring my endless amounts of stress, uncertain plans and spending more time traveling around the world instead of coming back home! I'll get a real job now I promise!

Last but certainly not least, I would like to thank Scotbeef Limited for funding this very interesting project and hence giving me the opportunity to do a PhD. I would like to thank late Ian Galloway, Suzy England, Laura Barwick for our great collaboration, for giving me numerous opportunities to engage with producers at many Scotbeef events and hence get a more realistic idea of the industry, for 'pushing' me for answers that would be relevant and have an impact for producers and the industry in general. I would like to thank Karol Wojcik for his time and efforts helping set up the abattoir sampling, the meat inspectors for their interest, cooperation and very informative input during the sampling and all the abattoir workers for really caring about my colleague's and my safety and for helping us get the job done by making our working environment pleasant and practical.

Publications and presentations

The following papers include work that has arisen from this thesis.

Sargison ND, Francis E, Davison C, Bronsvoort BMdeC, Handel I, **Mazeri S**. Observations on the biology, epidemiology and economic relevance of rumen flukes (*Paramphistomidae*) in cattle kept in a temperate environment. Veterinary Parasitology. 2016;219:7-16.

Mazeri S, Sargison ND, Kelly RF, Bronsvoort BMdeC, Handel IG. Evaluation of the performance of five diagnostic tests for *Fasciola hepatica* infection in naturally infected cattle using a Bayesian no gold standard approach. PLoS ONE. 2016; 11(8): e0161621.

Mazeri S, Rydevik G, Handel IG, Bronsvoort BMdeC, Sargison ND. Estimation of the delay in reaching slaughter weight in beef cattle infected with *F. hepatica* using different diagnostic measures. (Manuscript in preparation)

Mazeri S, Bessell PR, Sargison ND, Handel IG, Bronsvoort BMdeC. Risk factor analysis for liver rejection due to signs of *F. hepatica* infection in cattle slaughtered in Scotland. (Manuscript in preparation)

Work from this thesis has been presented in the following conferences/meetings

2015: International Society for Veterinary Epidemiology and Economics, Yucatan (oral and poster presentation)

2015: European College of Veterinary Public Health Annual Conference, Belgrade (poster presentation)

2015: British Society for Parasitology Autumn Symposium 'One Health: Parasites and Beyond', London (poster presentation)

2015: World Association for the Advancement of Veterinary Parasitology, Liverpool (2 oral presentations)

2015: Roslin Institute Research Student Day, Edinburgh (oral presentation)

2014: British Society for Parasitology Spring meeting, Cambridge (oral presentation)

2013-2014: Edinburgh Infectious Diseases Annual symposium, Edinburgh (poster presentations)

2013-2014: Roslin Institute Research Student Day, Edinburgh (poster presentations)

2013: Sheep Veterinary Society Autumn Meeting, Edinburgh (oral presentation)

2013: Society for Veterinary Epidemiology and Preventive Medicine Annual Meeting, Madrid (poster presentation)

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Table of abbreviations

AE: Abattoir employee

AIC: Akaike information criterion

AUC: Area under the curve

BCG: Bacille Calmette-Guérin

BCI: Bayesian Credible Interval

BCMS: British Cattle Movement System

BF: Blocking buffer

CB: Coating buffer

CCA: Complete case analysis

CI: Confidence interval

CRT: Coproantigen reduction test

CTS: Cattle Tracing System

DTM: Digital terrain model

ELISA: Enzyme-linked immunosorbent assays

ES: Excretory/secretory

FEC: Faecal Egg Count

FECRT: Faecal egg count reduction test

GIS: Geographic Information Systems

IFN: Interferon

IPCC: Intergovernmental Panel on Climate Change

LAMP: Loop-mediated isothermal amplification

LOCF: Last observation carried forward

MAR: Missing at random

MCAR: Missing completely at random

MCMC: Markov chain Monte Carlo

MD: Missing data

MHS: Meat Hygiene Service

MI: Meat Inspectors

MIM: Missing indicator method

MNAR: Missing not at random

NADIS: National Animal Disease Information Service

NDVI: Normalized Difference Vegetation Index

NGS: No gold standard

NPV: Negative predictive value

OD: Optical density

OMS: Overall mean/mode substitution

OR: Odds ratio

PCR: Polymerase chain reaction

PP: Percent positive

PPV: Positive predictive value

R: Researcher

ROC: Receiver operating characteristic

SCITT: Single comparative intradermal tuberculin test

SCOPS: Sustainable control of parasites in sheep

Se: Sensitivity

Sp: Specificity

TCBZ-R: Triclabendazole resistance

TCBZ: Triclabendazole

Th1: Type 1 T helper

UKCIP: UK Climate Impacts Programme

WB: Wash buffer

cELISA: BIO K201 Fasciola Coproantigen ELISA

p: Prevalence

sELISA: Serum antibody ELISA

Chapter 1

Introduction

1.1 Liver fluke - here to stay?

"*In view of the exceptional character of the weather during the past season, apprehension has been expressed in many quarters that the fluke parasite which causes liver-rot may increase to such an extent as to be a serious menace to the sheep stock of the country. The Board are accordingly considering what action they can properly take in the matter, in the way of collection and issue of information concerning possible preventive and remedial measures.*"

Board of Agriculture and Fisheries, December 1903 (1).

"*We 've had over 18 months where the liver fluke has had it all its own way; wet summers combined with mild and wet winters. The risk period is now much longer, normally we 'd see liver fluke from November to February but now we 're seeing it as early as late August and on into March*"

Lesley Stubbings, Sustainable control of parasites in sheep (SCOPS) group, May 2013 (2).

More than a century later UK farmers, veterinarians, epidemiologists and the state are facing similar issues. The parasite's evolving epidemiology along with its life cycle's high dependence on the changing climatic conditions are still making its control in UK cattle herds and sheep flocks a not so straightforward task. The following literature review will discuss the basic parasite biology, available diagnostic and control strategies as well why fasciolosis is possibly here to stay; even though there is great potential for reducing its prevalence and hence its effect on livestock production by understanding and improving our use of available tools relevant to its control.

1.2 Fasciolosis caused by *Fasciola hepatica*

Fasciolosis, otherwise known as liver fluke, is a clinically and economically important disease of cattle, sheep and other mammals. First reported in 1379, it was thought to be caused by the consumption of a bad herb leaf which destroyed the entire liver of the animal (3; 4). It has long since been known that the disease is caused by trematode parasites of the genus *Fasciola*. *F. hepatica* is the most common aetiological agent of fasciolosis in temperate regions including the UK and can also infect humans (5).

The disease has been described in detail in sheep, and presents in three different forms. Acute fasciolosis may lead to sudden death if metacercaria numbers exceed 5000, while death may be preceded by loss of condition and ascites in smaller infections. Sub-acute fasciolosis occurs more often when sheep are continuously infected with smaller numbers of metacercaria and presents as lethargy, anaemia, weight loss or even death. Chronic fasciolosis may also occur, which is characterized by severe weight loss, ascites and sub-mandibular oedema (5). Liver pathology differs in the acute and more chronic stages of the disease. In the acute and subacute stages the liver is enlarged, friable and haemorrhages may be seen. On the other hand, in the chronic stages, the liver is pale, firm and fibrotic with an irregular shape (6).

Cattle are less susceptible to the disease, which is thought to be due to the large size of the liver which leads to a greater functional reserve as well as its fibrous nature, therefore a higher number of metacercariae is required for clinical disease (7). The chronic form of the disease is the most important and can lead to weight loss, anaemia and hypoproteinaemia. Clinical signs are often mild and may present as loss of productivity, while in severe cases sub-mandibular oedema may be seen. Acute or sub-acute forms of the disease occur mainly in young calves, in cases of heavy infection. Unlike sheep, cattle can develop partial immunity with age and liver pathology also includes bile duct

calcification and gall bladder enlargement (5; 6).

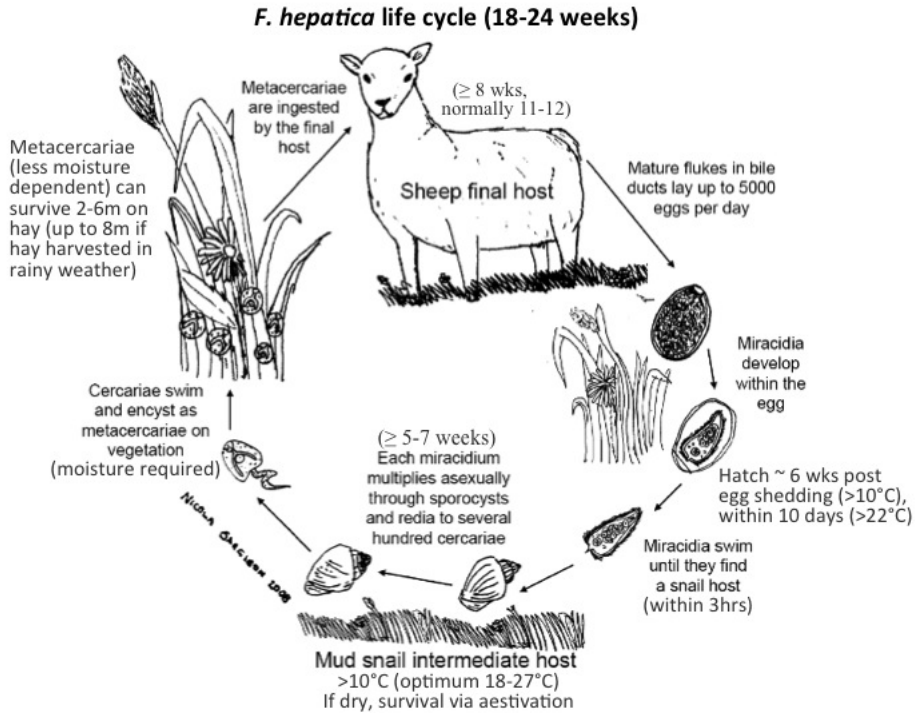


Figure 1.1: *F. hepatica* life cycle. Diagram by Nicola Sargison, adapted by the author (6; 8; 9).

F. hepatica has a complicated multi-host, highly climate dependent life cycle which normally takes between 18-24 weeks to be completed (Figure 1.1). Our knowledge about the "life-history" of liver fluke goes back more than a century and has been described in detail by Thomas (10) in 1883. Mammals including sheep, cattle, goats, deer and others are the final host and can be infected by ingesting metacercarial cysts on forage, suspended on soil or water while eating or drinking. After ingestion, metacercaria excyst and the juvenile flukes released penetrate the small intestinal wall, migrate through the peritoneal cavity and enter the liver by penetrating the liver capsule. This process takes one week. During the next 6-8 weeks, migration through the hepatic parenchyma occurs and flukes reach the bile ducts where maturation occurs. The prepatent period is usually 11 to 12 weeks, although egg production can start at 8 weeks post infection (8; 6).

Once eggs are excreted in faeces they can survive for a period of three weeks to several months depending on moisture availability. A temperature higher than 10^oC is required for miracidia development within the egg, a process that takes approximately six weeks, but can take less than two in temperatures higher than 20^oC. Upon hatching, miracidia need to find and penetrate a mud snail, the intermediate host, within 3 hours (max. 24 hours). Two researchers, Thomas (England) and Leuckart (Germany), independently confirmed *Galba truncatula*, previously called *Lymnaea truncatula*, as the first recognized intermediate host of *F. hepatica* in 1882 (10). *Galba truncatula* is considered the most common intermediate host of *F. hepatica* in Europe (6; 11). Within the snail, the miracidium transforms into a sporocyst and the growing sporocyst releases redia via asexual reproduction which in turn produce the final larval stage, the cercaria. This process takes a minimum of five to seven weeks but can take several months during unfavourable conditions for the flukes. Metacercariae exit the snail to swim and attach to firm surfaces, such as grass, where they encyst into the infective metacercarial stage. Encystment can also occur on water surfaces. Emergence from the snail also depends on temperature and moisture availability. Lastly, even though metacercariae are thought to survive for up to 3 hours in direct sunlight, there are reports of metacercariae surviving for up to 8 months in hay (12; 13; 14; 5; 6).

Temperature and moisture levels play an important role in the parasite's life cycle and it is generally accepted that average daily temperatures of more than 10^oC and high moisture levels are required for both the egg development and the reproduction of the parasite within the snail (3; 15). Other very important factors concerning the completion of the life cycle relate to the snail intermediate host itself. *G. truncatula* is a very adaptable organism that has an extremely fast reproduction rate, can survive for long periods in low temperatures by hibernation and in dry soil by aestivation and can tolerate a wide range of pH (3; 16).

Two distinct seasonal patterns of fasciolosis outbreaks have been described; summer and winter infection of snails, with the former being considered to cause more problems. Summer infection of snails arises when eggs deposited during spring and early summer, i.e. when moisture and temperature conditions become appropriate for egg and snail development, hatch and infect intermediate hosts. According to the life cycle described, this leads to encysted metacercaria appearing on pasture five to seven weeks later, leading to disease outbreaks during late summer and autumn. On the other hand, eggs deposited later on in the summer or early autumn will hatch and infect snails if appropriate conditions are available but may not be able to mature in the snail due to expected temperature decrease as winter is approaching. The parasite will therefore overwinter in the snail and development will resume when favourable conditions resume. Outbreaks due to winter infection will therefore precede summer infection and are expected to take place between mid summer and early autumn. The timing and intensity of winter infection will depend on the moisture levels during the first months that temperature is at favourable levels, usually May/June. If those months are wet, development of the parasite in the snail will be completed and excretion of cercaria will take place, while if dry snails will die off but no excretion of cercaria will take place (15; 17).

1.3 Host response to *F. hepatica*

Studies in sheep and cattle have described several immunomodulatory properties of *F. hepatica*. Within the first week of infection, activation of T helper 2 immune response has been described, with concurrent production of high titres of IgG1 antibodies (18; 19; 20; 21). At the same time downregulation of T helper 1 immune response has been observed in *F. hepatica* infections in both mice and cattle (22; 23; 24). In mice it is shown that the development of protective immunity is prevented by the ability of the

parasite to limit the T helper 1 immunre response, while enhancing T helper 2 mediated immune response and therefore promoting anti-inflammatory healing mechanisms (25; 26). As a result, chronic infection normally develops, with the exception of cases where overwhelmingly high levels of infection can lead to organ failure and therefore death.

Interestingly, development of partial immunity with age has been in cattle. However, this is thought to be due to mechanical obstruction due to bile duct calcification rather than innate and cellular immunity (5; 6). The lack of protection to re-infection was shown in a study by Clery et al. (27), where trickle infection with *F. hepatica* in previously infected cattle led to chronic infection despite the fact that T helper 2 response persisted in the blood. This lack of natural immune-mediated resistance to the parasite makes development of vaccines against *F. hepatica* more challenging (28), which will be discussed later.

1.4 Epidemiology of *F. hepatica* in the UK

The distribution of fasciolosis in the UK has changed considerably from a restricted distribution with occasional cases in the west of the country in the 1970s, to a widespread distribution and common outbreaks throughout the country. The last decade has seen even greater increases of fasciolosis incidence and, more importantly, changes in the distribution and timing of infection have been described. In the past, fasciolosis was most commonly seen in the wetter western regions of the country, while it is now evident that the disease has become endemic in the previously drier eastern regions (7; 29). These changes have been attributed to climate change, unpredictable weather conditions which make strategic treatment more difficult to plan, increase in animal movements and emerging treatment resistance concerning triclabendazole, the

only anthelmintic able to kill immature fluke one week post infection (30; 17; 31). Furthermore the spread of infection in areas and farms previously considered of low fasciolosis risk, meant that farmers were understandably unprepared having no control programmes in place hence suffering major losses.

1.5 Forecasting models

Due to the high dependence of the parasite transmission on climatic parameters, predictive models using information such as rainfall, number of rain days, evapotranspiration (15) or the number of wet days per month (32) have been around for a long time. The Ollerenshaw Index was published by Ollerenshaw in 1959 and it involves calculating the monthly risk of fasciolosis using the following formula (15):

$$Mt = N(R - P + 5)$$

Where *Mt* is the potential development of the parasite, *N* is the number of days with more than 0.2 mm of rain that month, *R* is the rainfall in inches that month and *P* is the potential evapotranspiration in inches that month. The original model used data only from May to October as temperatures during the rest of the year were prohibitive for appreciable development of the parasite. Additionally when estimating the total annual risk, *Mt* of months May and October was divided in half (15). The approach was first developed in Anglesey, but was then evaluated by Ollerenshaw himself using historic monthly reports of fasciolosis along with meteorological data during 1958-1962 in England and Wales and was found to be reasonably reliable in predicting broad trends of liver fluke incidence in the country (33). Since then, this approach has been widely used in the UK with various modifications. For example, the National Animal Disease Information Service (NADIS) uses the Ollerenshaw index to provide short-term forecasts for liver fluke risk. More precisely, the country has been divided into 10

regions and NADIS provides region specific forecast for each one of them. Scotland in particular is divided in to three regions; North West, East and Southwest (34).

More recently Geographic Information Systems (GIS) have been used for the development of forecasting models which can incorporate more detailed information on many fluke related factors such as meteorological data, soil information, Normalized Difference Vegetation Index (NDVI), host distribution and more in order to provide a dynamic visual representation of the infection risk which can be easily updated (35; 36; 37).

1.6 The effect of climate change on the risk of *F. hepatica*

According to the MetOffice, climate change is "a large-scale, long-term shift in the planet's weather patterns or average temperatures". This might involve higher temperatures, changes in rainfall, changes in seasons and more (38). The possible effects of climate change on the prevalence of vector-borne parasitic infections have been raised decades ago by the Intergovernmental Panel on Climate Change (IPCC) in their Climate Change Scientific Report published in 1990 (39). In their most recent report on climate change, IPCC state that we have had a successive increase in the Earth's surface temperature during the last three decades and that there was a warming of 0.85^{o}C of the global land and ocean surface temperature between 1880 to 2012. Furthermore, they report an overall negative impact of climate change on crop yields based on results of multiple studies from various regions. They also reiterate their previous concern that warming will lead to overall increased risks from vector-borne diseases, even though some areas will be too hot for vector survival (40).

Hughes et al. (41) has summarised evidence-based biological effects of climate change on living organisms which include effects on their physiology, development rate, distribution as well as effects on the timing and length of the life cycle. Furthermore, a number of recent studies have highlighted the causal relationship between climate change and changing prevalence of parasitic diseases (42; 43; 17). Fasciolosis is the vector-borne disease with the widest geographical distribution known, in terms of latitude and longitude and especially altitude (44). This reflects the great spreading power of the parasite which is related to the adaptability of the parasite in new intermediate and definitive hosts, as well as the great capacity of the snail intermediate host to reproduce and disperse widely (45). As described previously, the liver fluke life cycle depends heavily on climatic characteristics. For this reason liver fluke incidence is expected to be greatly affected by climate change, with varying climatic conditions affecting the development and survival of both the free living stages of the parasite and the stages within the intermediate host, as well as the reproduction and spread of the intermediate host. Increased temperatures are associated with significantly increased numbers of cercariae though accelerating their production as well as triggering their emergence from the snail (46). This is due to the increase in the intermediate host metabolic activity, resulting in increased energy availability for the parasite's development. Similarly, increase in rainfall can increase the time period and geographical area where both the free living stages of fluke as well as the snails can survive (44). On top of that, milder and wetter climatic conditions will increase the chance of overwintering of infected snails and prolong the survival of infective metacercariae on pasture (47).

In the UK, several studies have attributed the increase in prevalence and the changing distribution of animal fasciolosis to global warming. Mitchell (7), in his update on fasciolosis in cattle and sheep in Scotland, described the spread of the disease from the traditionally poorly drained pastures in the west to the traditionally drier regions in the

east of the country as well as the Scottish Borders, areas where fluke was previously not considered a problem. This has been attributed to the milder, wetter weather seen in Scotland between 1999 and 2001 compared to mean temperature and rainfall figures for 1961 to 1990. Similarly, Kenyon et al. (48) report that occurrence and timing of fasciolosis outbreaks in sheep in south eastern Scotland, are beyond the known patterns of disease. They support that this can be attributed to changing temperature and rainfall patterns and that these changes will, in the first instance, have serious implications in parasite control. Similarly, Pritchard et al. (30) described an increase of fasciolosis in cattle in East Anglia, an area where fasciolosis was previously considered a sporadic problem. They argued that climate change was one of the important factors for this increase due to an increase in the number of days with average temperatures greater than 10^oC as well as annual and summer rainfall.

Using adapted versions of the Ollerenshaw Index (15) and predicted climate data, Fox et al. (17) and Caminade et al. (49) have tried to estimate how predicted climate change will affect fasciolosis prevalence in the UK and Europe respectively. Northern Europe and the UK in particular, are predicted to face continued increase in fasciolosis incidence, while the season suitable for parasite development outside the mammalian host is expected to be extended. Caminade et al. (49). Fox et al. (17) have predicted unprecedented levels of risk in parts of the UK in the future and changes in the timing of disease outbreaks as the risk for overwintering larvae increases. For example, they predicted that serious epidemics will be a common occurrence in parts of Scotland and parts of Wales by 2020, and 2050 respectively. Despite predicting an overall long term increase of the risk of infection, some areas are predicted to have decreased levels of summer infection due to limited water availability.

Climate change is happening and is likely to be one of the main drivers of the changing epidemiology of fasciolosis in the UK. It is therefore very important to understand how

predicted climate change will affect the risk of fasciolosis in order to direct strategies for disease surveillance and control where they are most needed (50).

1.7 Risk Factors

Despite the fact that great focus has been placed on the analysis of the risk of *F. hepatica* infection associated with climatic conditions, the role of herd specific, management related risk factors, what Ollerenshaw and Rowlands call “constant factors" (15) has not been greatly explored. Figure 1.2 attempts to simplify the causal web of *F. hepatica* showing the interrelationship of the parasite’s life cycle, mammalian host and management related factors and the environment. Bennema et al. (51) sampled 1762 dairy farms in Flanders, Belgium for three consecutive years (2006-2008) and the association between *F. hepatica* infection levels (bulk-tank milk antibody ELISA) and meteorological, environmental and management factors was investigated. Annual rainfall, pasture mowing, grazing season length and proportion of grazed grass included in the diet were identified as significant predictors. Additionally, a related smaller scale, higher definition study by Charlier et al. (52) which also looked at snail related factors, identified significant associations between exposure to *F. hepatica*, as measured by bulk milk ELISA, and herd specific factors. These included the number of potential snail habitats, the presence of snails, drainage of pastures, stocking rate, the type of watering place and the month of turnout of cows.

McCann et al. (53; 54) estimated the seroprevalence of *F. hepatica* in dairy herds in England and Wales and used linear regression models to explain the distribution of the parasite based on information about climatic, soil and environmental parameters as well as the presence of suitable hosts and pasture types. The overall seroprevalence in England was estimated to be 72% with the lowest seroprevalence estimated in the

East (25.0%) and the highest in the North-west (95.2%). Factors found to explain the observed distribution included rainfall, temperature, soil pH, slope and poor land quality. Additionally, differences in seroprevalence were observed between closely located areas which may be explained by area specific environmental or farm management factors but this requires further investigation.

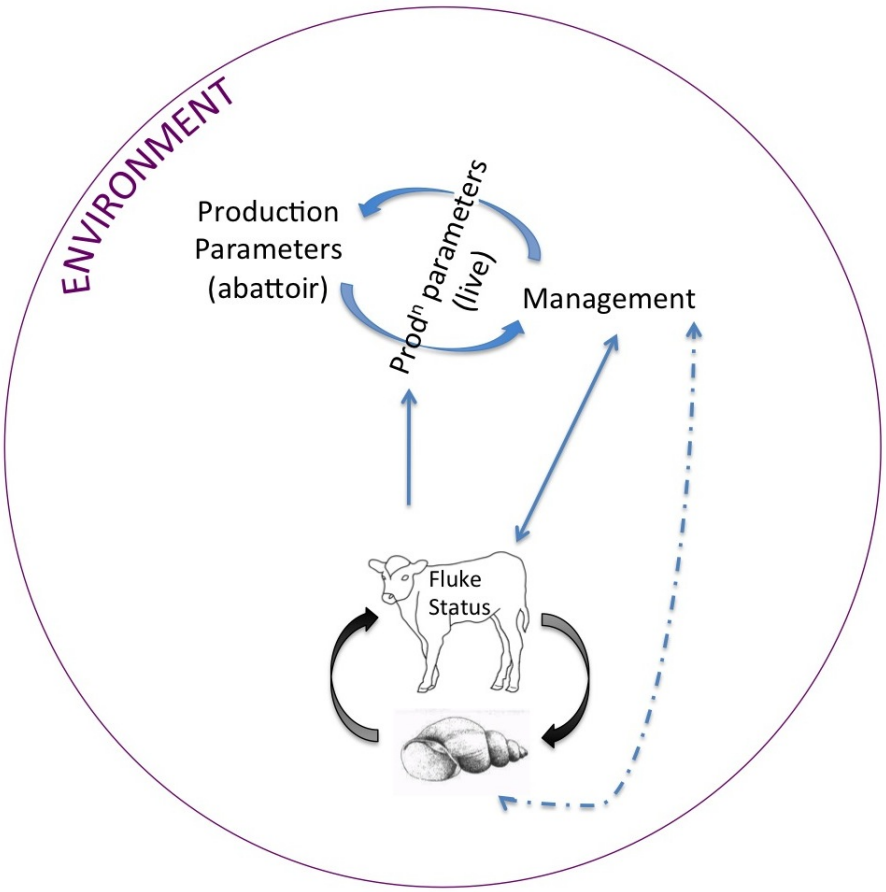


Figure 1.2: *F. hepatica* causal web. Due to the dependence of the extra-mammalian stages of the parasite's development on both climatic parameters and the existence of the intermediate host, understanding the risk factors associated with an animal being infected with liver fluke is complicated. The diagram represents a simplified causal web showing the interrelationship between the parasite's life cycle, the mammalian host, management related factors and the environment.

1.8 Economic Impact

In addition to its important clinical effects, especially in lambs and calves, fasciolosis can have significant economic impact. The worldwide cost of fasciolosis has been estimated to be around $3 billion (55). In the UK, the annual cost of fasciolosis to the cattle industry has been estimated to be £23 million (56), a figure based on crude estimates of earlier studies by Bennett et al. (57; 58). The real cost of fasciolosis in livestock is unknown, but it is likely to be much higher.

Kaplan (8) in his review of the economic impact of *F. hepatica* in cattle production explains the economic importance of the infection and the difficulty in measuring it due to the fact that it is usually caused by sub-clinical disease and results in indirect losses. Economic losses arise through the parasite's effects on productivity and include increased culling and reduced sale weights of culled cows, reduced reproductive performance and milk production as well as reduced weaning weights, growth rates and feed conversion rates. The effects of the infection in dairy herds have been studied by Charlier et al. (59) and include decreased annual milk yields and milk-fat % as well as increased inter-calving intervals. Furthermore, Sanchez-Vazquez and Lewis (60) have recently provided evidence to suggest that liver fluke rejection at the abattoir is associated with lower cold carcass weight, carcass conformation grade and fat levels. The lack of obvious clinical signs in cattle and the scarcity of information on liver rejection returned to the producers result to these losses commonly being attributed to other causes such as poor weather conditions or bad quality crops (61).

Similarly, fasciolosis in sheep can cause high barren rates, reduced twinning rates, longer lambing periods, ill thrift and death all of which have important economic impacts (62). Studies on the effect of liver fluke on ovine carcass characteristics are limited, possibly due to the absence of information on the infection status of individual

sheep going through abattoirs. Lastly, in both cattle and sheep there is the direct cost of increased liver rejections at the abattoir due to the effects of the infection on the livers and costs arising due to veterinary fees as well as treatment costs (8).

Accurately estimating the effect of *F. hepatica* infection on carcass characteristics of cattle and sheep is difficult and one needs to account for the complicated role of nutrition, which appears to have both a direct effect on carcass weight as well as through minimizing the pathogenic effects of the parasite (63). Consequently, further studies on the impact of *F. hepatica infection* on carcass characteristics of cattle and sheep are required and will need to take into consideration other factors affecting carcass characteristics such as animal health plans, nutrition and more (64; 65).

1.9 Co-infections

In addition to its direct impact on the animal's health and production, fasciolosis is also important due to its role in co-infections. Similar to other helminths, *F. hepatica* uses immunoregulatory strategies to manipulate the host's immune system in order to establish persistent infections (66). This downregulation of the immune system has been shown to also affect the host's response to other infections. For instance, *F. hepatica* co-infection has been shown to suppress the host's protective Type 1 T helper cells (Th1) response to *Bordetella pertussis* infection and delay bacterial clearance from the lungs of infected mice (22). Similarly, fasciolosis has been shown to increase cattle susceptibility to *Salmonella dublin* (67).

In the UK, it has been suggested that the high prevalence of fasciolosis in the country is associated with the failure of the current eradication programme against bovine tuberculosis (68). This is supported by earlier experimental studies which have shown that there is reduced Interferon (IFN)-γ responsiveness to both Bacille Calmette-Guérin

(BCG) (69) and virulent *Mycobacterium bovis* infections (24) in cattle with *F. hepatica* co-infection. Flynn et al (69) also suggested that the diagnostic performance of the two tests most commonly used for the diagnosis of bovine tuberculosis; the single comparative intradermal tuberculin test (SCITT) and the IFN-γ test, is compromised in these animals. A more recent study by Claridge et al (68) provided further evidence to support this hypothesis by showing that the magnitude of the SICCT tests is reduced in cattle experimentally co-infected with both pathogens (68). The authors also showed that exposure to *F. hepatica* is associated with reduced odds of diagnosing bovine tuberculosis on a dairy farm, using data from 1821 dairy farms, i.e. providing evidence that experimental study results might also apply to naturally infected cattle populations (68).

1.10 Diagnostic tests

The best method for diagnosing fasciolosis is post mortem examination of untreated animals (7). This is of course not always practical or possible and various laboratory methods are available for the diagnosis of fasciolosis in the live animal. A list of the main available diagnostics can be found below.

1.10.1 Biochemistry and haematology

Blood samples can be obtained for biochemistry and haematology analysis to support clinical diagnosis of liver fluke infection (70). Liver enzyme concentrations such as aspartate aminotransferase and glutamate dehydrogenase may be increased as early as two weeks post infection and are supportive of an acute liver fluke diagnosis in sheep. Increased L-gamma glutamyl transferase concentrations can indicate the presence of adult flukes in the biliary tree during chronic infection. Peripheral eosinophilia can be

a useful indicator of disease in cattle (7). Results of these tests are only indicative and involve blood sampling which is an invasive method for diagnosis.

1.10.2 Faecal egg count

The Faecal egg count (FEC) test has been around for a long time and various protocols exist. It involves diagnosis of *F. hepatica* infection by microscopic detection of eggs in a weighted amount of faeces using either flotation or sedimentation techniques and providing a number of eggs per gram (71; 72; 73).

The test is simple to perform and read and requires minimal technical equipment. This makes it very accessible hence it is the test most commonly used for the diagnosis of fasciolosis in cattle and sheep. Nonetheless, FECs have two important limitations. First, by definition, the test can only diagnose patent infections as only adult parasites will produce eggs, therefore pre-patent infections will be missed (74). Secondly, it is generally accepted that it has a low sensitivity, i.e. it detects a low proportion of truly positive animals, and this varies depending on the protocol used (75; 76). On the other hand, FECs are believed to have a specificity close to 1, i.e. if an egg is found the animal is very likely to be truly infected. The only other parasite eggs that look very similar to *Fasciola* fluke eggs (even though they have a different colour) are those of *Paramphistome* flukes. The recent increase in *Calicophoron daubneyi* rumen fluke infections in the UK might therefore compromise the test's specificity (77).

1.10.3 Serum antibody enzyme-linked immunosorbent assays (ELISA)

The detection of antibodies in serum using ELISA based tests for the diagnosis of *F. hepatica* has been around for many years. Various commercially available as well as

in-house antibody ELISAs have been developed using a wide range of antigens including excretory/secretory antigens, tegumental antigens, cystein proteases and somatic antigens. The reported sensitivities and specificities of these methods for the diagnosis of fasciolosis range from 91.7% to 100% and 94.6% to 100% respectively (70). The advantage of using a serum antibody ELISA for the diagnosis of fasciolosis is that it can potentially detect infection as early as two weeks post infection. Nevertheless, due to the very nature of the test it cannot reliably distinguish between current and previous infections (78; 79; 80) even though antibody levels have been reported to decrease after a variable amount of time post treatment (81; 82). Lastly, requirement of a blood sample renders serum antibody ELISAs an invasive test.

1.10.4 Copro-antigen ELISA

Obtaining a blood sample for the diagnosis of fasciolosis is an invasive technique and, while collection of faecal samples is much more practical, the limitations of FEC have already been discussed. The detection of antigen in faeces has been investigated by various groups using different capture antibodies (70). In 2004, Mezo et al. (83) presented a new copro-antigen ELISA for the diagnosis of current fasciolosis in sheep and cattle. The ELISA uses the MM3 monoclonal antibody to detect *F. hepatica* excretory/secretory antigens (84) in faecal samples and is now commercially available by Bio-X Diagnostics. It was firstly reported to have a sensitivity of 100% in detecting sheep with a fluke burden of 1 or more and cattle with fluke burden of 2 or more, while it was reported to have a very high specificity with no cross reactivity with parasites like *Moniezia*, *Dicrocoelium*, *Echinococcus* and *Paramphistomum cervi* (83; 85). The great advantage of this test over FEC and serum antibody ELISAs is that it can detect early stages of infection without the limitation of giving positive results due to past exposure (83; 86). Nevertheless, Gordon et al. (31), in a study of naturally infected

lambs, reported that the test detected infection at the same time point as FEC, highlighting the possibility that the test performs differently when used in experimentally challenged animals compared to naturally infected ones. Similarly, two studies have reported lower sensitivity estimates for the performance of the test in cattle challenging the initial results (76; 87). The copro-antigen ELISA presents a good alternative to FEC, but being a fairly new test it requires further evaluation.

1.10.5 DNA-based techniques

DNA-based methods have not been used for the clinical diagnosis of *F. hepatica* infection, even though polymerase chain reaction (PCR)-based methods have been used in research for genetic characterization, identification or differentiation of *Fasciola* spp. (70). Nevertheless, a recent study has shown promising results using a nested-PCR on faecal samples of experimentally and naturally infected sheep. The study suggested that the method can detect infection as early as two weeks post infection i.e. earlier than both FEC and the commercially available copro-antigen ELISA, has a higher sensitivity than FEC and is highly specific (88). These results are very promising, but the time, cost, expertise and technical equipment required make these methods less applicable for clinical diagnosis.

The loop-mediated isothermal amplification (LAMP) assay was first presented in 2000 by Notomi et al. (89) who described it as a gene amplification technique that is very specific, efficient and rapid and can be run under isothermal conditions. Two recent studies have presented promising results for the application of LAMP assays for the diagnosis of *F. hepatica* in faeces reporting results comparable or even more accurate than conventional PCR techniques and taking a much shorter time to obtain results (90; 91). The fact that LAMP is faster, can be run at a constant temperature and results can be read with the naked eye provide a good basis for future development of

a pen-side test, which may be much more applicable for use in clinical diagnosis of fasciolosis (92).

1.11 Parasite control and available anthelmintics

The complex life cycle of *F. hepatica* provides various opportunities for parasite control with the main aim being to decrease the risk of infection of the final host by minimising metacercarial challenge. A successful control program should not depend solely on flukicide drugs. For example, grazing management strategies can be used to avoid heavily contaminated pastures or fencing off of high risk pastures and drainage -in areas where it is environmentally acceptable- could be used to make pastures less favourable for survival of both snails and the free living stages of the parasite (29). Nevertheless, chemotherapy remains one of the main ways to control fasciolosis. Since no molluscicides are licenced for use in the UK, the chemical control of fasciolosis in livestock depends solely on anthelmintic drugs. There is a range of anthelmintics used for the treatment of fasciolosis in livestock in the UK and these vary in efficacy, price and safety withdrawal periods. There are several products available on the market either including single ingredients or combinations with other flukicides or anthelmintics effective against nematodes. Flukicides are derived from three different groups of anthelmintics which are listed below:

1.11.1 Benzimidazoles

Benzimidazoles used in the treatment of fasciolosis include triclabendazole and albendazole. In general the mode of action of benzimidazole compounds involves binding to the parasite's tubulin, a protein found in microtubules, plasma and mitochondrial membranes. This results in parasite starvation, by inhibiting glucose uptake,

protein secretion and production of microtubules. Nevertheless, the mode of action of triclabendazole is poorly understood although there is evidence to support that it also targets tubulins (50; 93). Triclabendazole has been the drug of choice for treating fasciolosis in livestock for more than 20 years as it is the only flukicide with practical efficacy against parasites less than five weeks post infection (94; 95). Albendazole on the other hand is only effective against adult parasites (96).

1.11.2 Salicylanilides and substituted phenols

Three compounds are available in this category; closantel, oxyclozanide (salicylanilides) and nitroxynil (a substituted phenol). They are extensively bound to plasma proteins, which is thought to be the reason why they are not effective against early immature flukes that possibly feed mainly on liver tissue. Nevertheless, closantel and nitroxynil are thought to be effective against late immature flukes (29). This is thought to be because as flukes grow and migrate through the liver, the haemorrhage caused results in consumption of the drug by the parasite (93).

1.11.3 Benzine sulphonamides

Clorsulon is a benzene sulphonamide compound effective at treating adult fluke in cattle only (97).

1.12 Anthelmintic resistance

The first case of triclabendazole resistance (TCBZ-R) was reported on a sheep farm in Australia in 1995 by Overend and Bowen (98). Since then, TCBZ-R has been reported

on at least 30 properties in several European countries, Australia, New Zealand and Latin America (99).

In the UK, TCBZ-R has been reported as an emerging problem of the western regions (29). The first two cases of resistance were reported in Scotland and Wales in 1998 (100) and 2000 (101) respectively. Since then, Sargison and Scott (29) reported TCBZ-R in a sheep flock in south-East Scotland, Daniel et al. (102) reported strong indications of resistance in 7 sheep farms (6 in Wales and 1 in Scotland), while Gordon et al. (103) confirmed diagnosis of TCBZ-R in sheep in Dumfries and Galloway, Scotland using a dose and slaughter trial. Lastly, resistance was also recently reported on two farms in N. Ireland (104) while Mooney et al. (105) reported potential TCBZ-R in a study on the efficacy of four anthelmintics using the Faecal Egg Count Reduction test in a hill sheep flock in neighbouring Ireland.

There are no clear guidelines for the diagnosis of TCBZ-R in live animals, and definite diagnosis of TCBZ-R requires a treatment and slaughter trial (106). This is clearly not a practical approach to use in commercial flocks, but it could be a useful exercise on sheep that die post treatment (29). Commonly used approaches include a Faecal Egg Count Reduction Test (FECRT) (107) and more recently, the coproantigen reduction test (CRT) (86). The FECRT is commonly used for the evaluation of the efficacy of drugs against nematode infections by testing faecal samples pre and 14 days post treatment (106). Its use for the diagnosis of TCBZ-R has several important limitations. Triclabendazole is commonly used to treat pre-patent infections, while FEC can only detect patent infections. Furthermore, fluke egg shedding is often irregular and can continue for a variable amount of time post successful treatment as eggs may be stored in the gall bladder (108; 86). Even though FECs are quite straightforward to perform, it is a time consuming procedure. For this reason, Daniel et al. (102) developed a composite FECRT where samples of a group of animals can be tested together, with

similar sensitivites to the original FECRT. The copro-antigen reduction test involves measuring coproantigens in faeces pre and post treatment using the commercial copro-antigen ELISA (Bio-X Diagnostics in Belgium) presented by Mezo et al. (83). The protocol, standardised by Flanagan et al. (86), recommends a repeat test of copro-antigen ELISA samples 14 day post treatment using a negative result as an indication of treatment success. The strength of this protocol mainly lies in the fact that copro-antigen can be detected 2.5-3 weeks earlier than eggs appear in FECs (86).

Failure to obtain a negative test result post treatment is not necessarily due to TCBZ-R. In fact underdosing as a result of poor weight estimation, use of faulty drenching equipment, poor drug storage or use of inferior quality products is a common cause of apparent drug failure (50; 29; 109). Fairweather (50) raises concerns over this overdiagnosis of TCBZ-R arguing that this will encourage farmers to unnecessarily alter their choice or time of drug administration resulting in compromised anthelmintic control. On the other hand, Sargison and Scot (109) argue that identifying triclabendazole inefficacy for whichever reason encourages farmers to use preventive management strategies such as grazing management and more strategic timing of drug administration due to the raised awareness of the ineffectiveness of whole flock metaphylactic treatments, which in the long run might improve fasciolosis control and decrease the probability of emergence of TCBZ-R.

The mechanism by which TCBZ-R develops is not clear. Nevertheless, several studies have provided convincing evidence that altered drug uptake and altered drug metabolism play a more important role than tubulin mutations. Understanding the exact mechanism of resistance might help improve treatment of animals infected with TCBZ-R liver fluke either through modulation of the current TCBZ compound or by administration of combinations of TCBZ and inhibitors (50; 99). Currently, control lies in the use of other flukicides or combination therapies. This can be problematic for two

reasons. Firstly, no other drug can treat very early stages of infection and secondly, though much more uncommonly, resistance has already been reported in Albendazole, Clorsulon, Closantel and Nitroxynil (99). A more sustainable solution for reducing the emergence of anthelmintic resistance therefore would be to increase the reliance on using pasture management strategies to reduce the risk of fasciolosis and enhance the monitoring of treatment efficiency.

1.13 Vaccination

Development of effective vaccines commonly depends on the understanding the host's immune response (110). Nevertheless, the lack of evidence for natural innate and cellular immune-mediated resistance to *F. hepatica* in both cattle and sheep makes vaccine development more difficult (28). Sheep do not develop acquired immunity to the infection (111) and parasites are reported to survive in the animal for as long as 11 years (112). On the other hand, some evidence of resistance to the parasite has been reported in cattle, where most flukes are expelled within 7 months post infection. Nonetheless, this is likely to be due to the cattle specific physiological response to infection which involves liver fibrosis and bile duct calcification and might lead to parasite rejection (110).

There are currently no commercially available liver fluke vaccines. Several antigens have been identified that can potentially be included in vaccines either alone or in combinations and these include fatty acid binding proteins, two cathepsin L peptidases (L1 and L2), leucine aminopeptidase, glutathion S-tranferase and thioredoxin peroxidase (113; 114). An extensive list of vaccine trials in both sheep and cattle published by Toet et al. (110) shows that no vaccine has shown 100% efficacy (reduction in adult flukes in comparison to unvaccinated controls) against liver fluke infection so far, al-

though partial protection has been reported. The two major pitfalls on the current vaccine research are the lack of repeatability of results, which is partly attributable to the lack of a standardized vaccine protocol and the lack of field trials to show the actual effectiveness of these vaccines (110; 28).

Various authors have challenged the need for a 100% efficient vaccine and emphasised the fact that even vaccines with sufficiently high partial efficacy could provide economic benefits especially in situations where there is high risk of TCBZ-R (110; 28; 115). Turner et al. (115) have built a mathematical model to assess the effectiveness of potential liver fluke vaccines under simulated field conditions in order to identify which level of efficacy would make a vaccine useful. Results suggest that the most important vaccine attributes include reduction in fluke fecundity which will reduce egg output, increase of immature fluke death rate, which will reduce burden within a season, and most importantly a vaccine should protect at least 90% of the animals during the whole season. On the other hand, increase in fluke maturation time does not seem to have a useful effect. In essence they have shown that potential candidate vaccines under field conditions can reduce total fluke burden by as much as 43% and daily egg output up to 99%, supporting the fact that they could contribute significantly to fasciolosis control (115).

Overall, in the absence of new anthelmintics development, commercialisation of partially effective vaccines might be a good short term solution in combating the increasing levels of fasciolosis in the face of emerging anthelmintic resistance, but more field studies and standardised vaccine protocols are required to ensure repeatability of current vaccine candidates efficacy results.

1.14 Fasciolosis in humans

Human fasciolosis has a worldwide distribution and is estimated to affect 2,4 to 17 million people, which is probably an underestimate since the levels of infection in large parts of Africa and Asia are unknown (116; 117). No deaths directly associated with human fasciolosis have been reported to date, which is possibly the reason why fasciolosis is one of the most neglected tropical diseases (118). In fact it has recently been included in the World Health Organisation of Neglected Tropical Diseases (119), among other important zoonoses such as Rabies, Echinococcosis, Leishmaniasis, Schistosomiasis, Onchocerciasis and more (120).

Similar to animals, humans become infected by ingesting cercariae in contaminated water or encysted metacercariae when consuming contaminated vegetables and the disease is most commonly seen in farming communities in low income countries (121). Nevertheless, the epidemiology of human fasciolosis is not necessarily in parallel to animal fasciolosis, as it is associated with specific habits and travelling. Sources of human infection include ingestion of freshwater plants, use of contaminated water to drink or to wash kitchen utensils and less commonly ingestion of raw liver infected with metacercariae (116).

The clinical presentation of fasciolosis in humans has four recognised phases. The incubation phase is the period between infection and appearance of the first clinical signs and can take as little as a few days to two to three months or longer. The acute phase follows, where liver destruction by migrating larvae can cause fever, abdominal pain, gastrointestinal disturbances and more. This phase can last between two to four months. The patient then moves to the latent phase which can last for months or years before signs of the chronic or obstructive phase appear due to the mechanical obstruction caused by cholangitis, cholecystitis and the space occupied by adult parasites

(122), which include discomfort, fever, hepatomegaly, nausea and more (116).

Fasciolosis, in humans is mainly of zoonotic origin. On top of that, the drug of choice for treatment of human fasciolosis is triclabendazole (TCBZ), i.e. the main drug used in livestock animals, for which, as mentioned earlier, resistance has been reported in various parts of the world (123; 99). It is therefore important that a one health approach is in place both to decrease levels of human infection as well as to limit emergence of TCBZ resistance.

Despite the fact that human fasciolosis is a problem in other European countries with high levels of livestock infections such as France, Spain and Portugal (44; 116), there is no evidence of indigenous zoonotic transmission of *F. hepatica* in the UK. Cases of human fasciolosis are seen sporadically and are almost always in patients who have travel histories to areas where fasciolosis is endemic (124).

1.15 The importance of the use of slaughterhouse data in epidemiological research

Slaughterhouse data have been long recognized as a valuable yet underutilised source of information for use in epidemiological research for both surveillance purposes, and to formulate epidemiological hypotheses (125). Their value lies in the fact that they can provide information about vast numbers of animals, commonly originating from large geographical areas and over long periods of time. Nowadays, slaughterhouse data including animal characteristics, carcass characteristics as well as information on offal or whole carcass rejections are often recorded electronically making the availability and usability of such datasets even easier. This information is systematically collected and provides a cost-effective resource. Furthermore, abattoirs themselves

provide an ideal place for carrying out epidemiological studies since large numbers of animals go through every day from multiple consignments and post-mortem sampling is potentially less technically challenging and comes with fewer ethical concerns than ante-mortem sampling. Information from such studies can be combined with and enhance routinely collected abattoir data.

However it is very important to bear in mind that slaughterhouse data come with several important limitations. Firstly, disease information collected can only relate to prevalence estimates (125). This is because there is no way to tell at which point in time the animal was infected. Secondly, meat inspection is generally considered to have low sensitivity (126) and its inaccuracy may vary between slaughterhouses or even between meat inspectors at the same slaughterhouse. Furthermore, sensitivity of passive surveillance, such as meat inspection, depends on disease awareness and is therefore expected to be higher for endemic diseases such as liver fluke compared to more rare infections (127). Inaccuracy of abattoir data can arise not only due to the imperfect sensitivities and specificities of meat inspection techniques, but also due to the speed of the abattoir line which dictates the time available for carrying out and reporting meat inspection, as well as the systems in place for data recording.

When describing the prevalence of a syndrome or disease in a population based on abattoir data there are several potential sources of bias that are important to have in mind. The age structure of slaughtered animals is likely to be different to the age structure of the general livestock population (125). Additionally, when trying to estimate the prevalence of the infection in different geographical regions there are two potential problems; a) different numbers of animals will reach the abattoir for each region resulting in regions not being evenly represented and b) low sample sizes for some regions will inevitably result in either very high or very low prevalence values (128). Acknowledging the aforementioned limitations of abattoir data, one can account for

them using appropriate statistical methodology and careful interpretation of results, to efficiently utilise this invaluable resource and gain important information on disease epidemiology and control (129).

Fasciolosis is an infection commonly recorded in abattoir data in the UK and most of Europe. According to Regulation (EC) No 854/2004, it is compulsory to inspect all livers of cattle slaughtered in abattoirs in Europe for signs of liver fluke infection. Abattoir data can therefore provide a very useful tool for improving our understanding of the changing epidemiology of liver fluke in the UK. The only available estimate of the sensitivity of abattoir liver inspection to detect current *F. hepatica* infection comes from a study by Rapsch et al. (75) in Switzerland who estimated it to be 63.2%. This was done by comparing abattoir liver inspection results from 1,331 cattle slaughtered at two different abattoirs in Switzerland to results of faecal egg counts, a serum antibody ELISA and gall bladder egg counts from the same animals (75). However, the accuracy of meat inspection is likely to vary between countries, as the epidemiology of infection might be different. When studies use abattoir data to estimate disease prevalence and the accuracy of meat inspection is unknown, it is important to acknowledge this or even try to estimate it.

The red meat industry (cattle, sheep, pigs) is one of Scotland's major economic contributors. In 2015 the output from the red meat industry alone was £1.18 billion, contributing 40% of the total agricultural output in Scotland. Beef production is the largest sector of Scottish farming and has contributed almost 29% of the Scottish agricultural output, and remains of much greater importance in Scotland when compared to the UK (13.6% of agricultural output) and the EU (8.5% of agricultural output). Furthermore, 1,736,100 head comprised the Scottish cattle population in 2015, of which 424,500 were beef breed herd animals (female cattle more than 2 years old with offspring). The average herd size (beef cows) per holding is 47 while the presence of large cattle

enterprises in Scotland means that almost half of the beef population belongs to 13.5% of the holdings. In total 459,150 cattle were slaughtered at Scottish abattoirs in 2015 (397,650 prime cattle and 61,500 older animals), of 371 kg prime cattle average carcass weight (steers - 392 kg, heifers - 344 kg and young bulls - 359 kg). The annual average price for prime cattle was 362p/kg dead weight and the average price for a steer was £1,417.80 (130).

Scotland has 24 licensed red meat abattoirs which operated during 2015, 20 of which processed cattle. Despite that, 71% of the cattle were slaughtered in the 5 largest abattoirs (130). Scotbeef limited (http://www.scotbeef.com), the main funding provider of this PhD, is Scotland's largest red meat manufacturer and processes around 110,000 cattle and 750,000 lambs per year. It is located just outside Stirling, at the Bridge of Allan but attracts producers from the North of England to the Orkney Islands. Data from Scotbeef cover a large number of producers throughout the country and provide a potentially valuable yet underutilized data source for the epidemiology of animal diseases. The current study analyses data collected at the abattoir in conjunction with other relevant data sources and data collected during abattoir based sampling, using robust statistical analyses to provide an insight into the changing biology of the parasite.

1.16 Main research question

How can abattoir information be used to investigate the changing epidemiology of *F. hepatica* in the UK and identify risk mitigating management strategies against fasciolosis?

1.17 Aims and Objectives

1. Evaluate the diagnostic sensitivity and specificity of serum antibody and copro-antigen ELISA tests, Faecal Egg Counts and meat inspection using abattoir based sampling
2. Estimate the difference in slaughter age between beef cattle infected with liver fluke vs. uninfected cattle using different measures of infection
3. Investigate the quantitative use of diagnostic tests in distinguishing between animals of high and low levels of infection
4. Estimate the association between climatic, environmental and management factors and *F. hepatica* infection

Chapter 2

Materials and methods

2.1 Introduction

This thesis used data routinely collected at the Scotbeef Limited Abattoir (Bridge of Allan) in combination with other data sources on climatic and environmental data. Additional data were collected through abattoir based sampling and questionnaires administered to producers. This chapter will provide an outline of the main data sources used in this thesis, the methodology used for abattoir based sampling, the diagnostic tests employed to process the samples and the producers questionnaire. Statistical methodology and specific data used will be described separately in the materials and methods section of each data chapter.

2.2 Data sources

2.2.1 Data routinely collected at Scotbeef abattoir

Figure 2.1 shows the data sources used in this project. Data are divided into two categories, those related to the producer and those related to individual cattle. When a producer wishes to start working with Scotbeef a registration document has to be completed. This contains information about their address, the type of farming, breeding and feeding as well as number of stock and the environment the farm is located in. All Scotbeef producers are required to be farm assured. Further to that an audit is conducted every 12-18 months to check whether each farm is eligible for selling meat to Marks and Spencer. Lastly, when a producer sends animals in he or she has to complete a movement document with information about the animals to be sent and a feeding declaration form. This is either done online or a hard copy of the form is completed. A recent addition to the cattle movement document, directly relevant to this project, has been the collection of information on when each animal was last treated for fluke and what with. Lastly, no cattle can be slaughtered if it is not accompanied by the correct passport.

In the slaughterhouse, information is collected on reasons for offal rejection along with production parameters including carcass grade and weight. In the lairage, cattle are mixed, hence they are not separated in batches by producer when they enter the slaughter line. Each animal can be uniquely identified by their eartag number which corresponds to a kill number which starts from zero every day. Therefore information collected at the grading stage for each kill number can be related to a specific passport number. At the meat inspection stage the offal is separated from the carcass, but a counter is in place to enable each offal inspection to correspond to a specific kill number. This enables liver fluke information to be recorded at the individual animal

level.

Liver inspection is routinely carried out at the abattoir by the Meat Hygiene Service (MHS). According to the manual for official controls, liver inspection requirements include visual inspection, palpation and incision of the gastric surface of the liver (131). Livers with signs of liver fluke related pathology then have to be rejected. At Scotbeef, since 2012, MHS decision regarding liver rejections is recorded as 'Active', 'Historic' or 'No fluke'. 'Active' is roughly defined as livers in which parasites were seen, while 'Historic' describes livers with liver fluke related pathology but no signs of current infection. Both 'Active' and 'Historic' livers have to be rejected. This is unlike most UK abattoirs and for the purposes of this thesis the standardised classification was used, i.e. 'Active' and 'Historic' livers were considered as liver fluke positive and 'No fluke' livers as liver fluke negative.

Scotbeef has two secure web based databases: BeefTrack and LambTrack, where information collected at the meat inspection and the grading stages, as described above, is stored for each producer. Every producer has an online account with information regarding their farm and animals slaughtered. Using their online account, they can organise when their animals will be slaughtered, obtain feedback on the performance and rejection status of their animals and more. Moreover, this is where data provided for this project are stored.

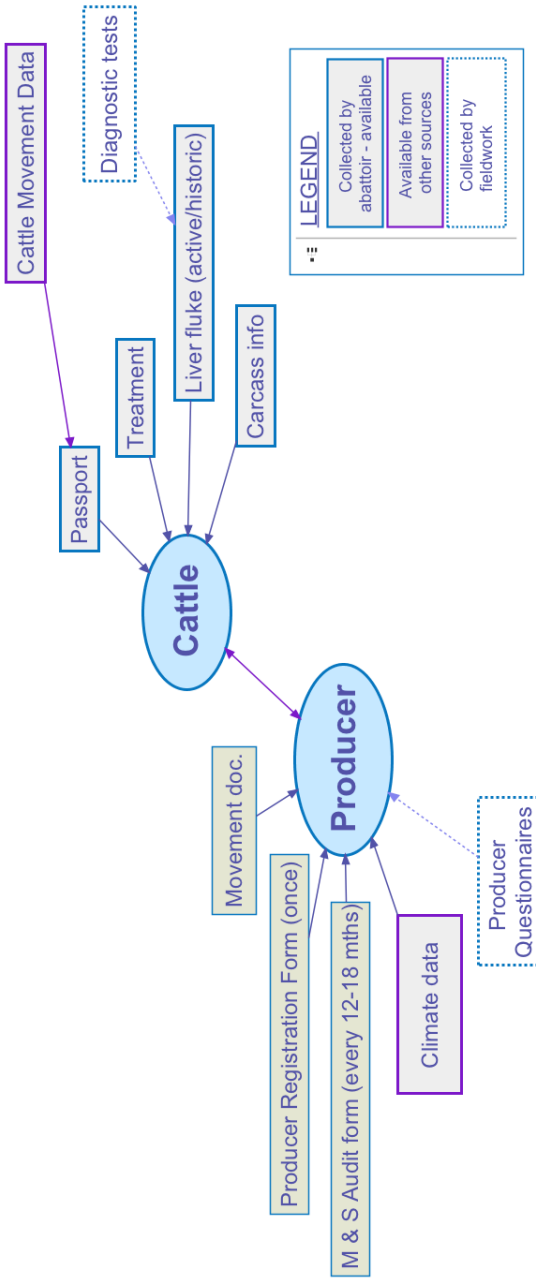


Figure 2.1: Diagram of data relevant to this project.

2.2.2 Cattle Tracing System database

All cattle in the United Kingdom are identified by their unique eartag number and have a passport issued by the British Cattle Movement System (BCMS) containing information on animal specific details such as sex, breed, date of birth and death and any movements that occurred throughout the animal's life (132). This information is held within the BCMS Cattle Tracing System (CTS) database (133). The eartag information collected at the abattoir, enabled us to link these two datasets and therefore extract data on the sex of each animal from the CTS database.

2.2.3 Climatic and environmental data sources

Soil type and pH data were sourced from the National Soil Map of Scotland which was created by the Macaulay Institute using data collected between 1947 and 1981 (134). UK Climate Impacts Programme (UKCIP) 5 km grid mean monthly data for rainfall and temperature between 2007-2011 were obtained from the UK Meteorological Office (135; 136). River data were extracted from the OS Open Rivers dataset sourced from EDINA (137) and this enabled us to calculate the distance to the closest river for each holding of origin. Slope and elevation data were extracted from the OS terrain 50 digital terrain model (DTM) data downloaded from EDINA (138). Farm area, as reported in the questionnaire described below, was used to construct a circle around the geographic coordinates of each farm, for which a mean slope and elevation was calculated using the 50m DTM.

2.3 Farm management questionnaire

Information about the type of farm and management strategies used on each farm was collected through a questionnaire. A list of relevant management information was created based on known and suggested risk factors for liver fluke infection in cattle as well as relevant questionnaires used in other studies of *Fasciola* and nematode parasites in Europe (139; 51; 52; 9). These were then turned into epidemiological questions relevant to UK farm management. SurveyMonkey® (Palo Alto CA, USA), an online questionnaire tool, was used to design both online and paper versions of the questionnaire. The questionnaire contained questions about the following: a) general farm details, b) respondent's details, c) type and numbers of animals kept, d) management of bought in animals, e) presence of other animals other than cattle on the farm, f) grazing management, g) pasture management, h) liver fluke management, and i) liver fluke history. The complete questionnaire can be found in the Appendix A.

Before administration the questionnaire was piloted in two phases. Firstly, paper versions were used to pilot the questionnaire with six producers. The producers were asked to complete the questionnaires alone, but to ask when something was unclear. In such cases, they were asked to suggest a better way of asking that question. Some questions were deemed unnecessary or too complicated to answer and were removed. The questionnaire was then adjusted accordingly and was further piloted with two producers using the online version. This was both to check the adjusted version of the questionnaire and the online version. Minimal changes were made after this phase and the questionnaire was finalised for use in the study. Producers taking part in the pilot study were chosen to represent different management systems and were within two hours drive from the city of Edinburgh as a matter of convenience.

The finalised questionnaire was administered through BeefTrack, Scotbeef's secure

web based database, through each producer's account. Between the 20th of February and the 4th of June 2014, a landing page appeared when a producer logged into the system where pending questionnaires were shown. A producer could choose to either complete the questionnaire or skip this page and move onto the system. Email reminders including web links to the questionnaires were also sent to producers once. Lastly, paper versions of the questionnaires were administered to producers who had not completed the online versions during the Marks and Spencer audit visits. Scotbeef producer codes were used in all modes of administration of the questionnaires to anonymise and uniquely identify each producer.

2.4 Abattoir based sampling

During sampling we collected each sampled animal's unique eartag number which for the purposes of this study enabled us to obtain data on the sex of each animal extracted from the CTS database described above.

2.4.1 Sampling overview

Samples were collected during three sampling periods. Sampling period A (June-July 2013) will be referred to as "summer 2013". Sampling period B (January-beginning of March 2014) will be referred to as "winter 2014". Lastly, sampling period C, which took place between the end of August 2014 and October 2014 will be referred to as "autumn 2014". Each period consisted of six sampling days, one per week and 32-36 animals were sampled each time. The day and number of animals sampled each day were constrained by logistics. We used systematic sampling, collecting samples from one cattle in every 10 slaughtered to allow time for processing and to represent animals slaughtered during the whole day. Animals to be sampled were clearly labelled at the time of bleeding and labels were maintained at all sampling stages to ensure that the correct samples were taken. Samples included blood, faecal samples as well as whole livers and gall bladders from each animal. Whole livers and gall bladders were stored at 4 °C and were analysed within 72 and 96 hours respectively. Blood samples were stored at 4 °C for 24 hours before sera were obtained and stored at -20 °C. 2g of faeces were stored at -20 °C, while the rest was stored at 4 °C for egg counting which took place within a week post sampling. Figure 2.2 provides an overview of the sampling procedure.

2.4.2 Sampling stations

Sampling included three sampling stations as described below, hence two to three people were required for each sampling day. As the members of the sample collection team were constantly changing, the author was responsible to explain the procedure before every visit to any new members.

Blood collection station

Blood collection took place in cooperation between the abattoir employee (AE) responsible for scanning cattle ear tags and the researcher (R). AE collected blood by placing an open 100 ml universal tube on a metallic stick and under the bleeding animal immediately after sticking. When the tube was full, AE handed the tube to R. The tube was pre-labelled with kill number by R. AE placed a tag with elastic band on the left forelimb above the carpal joint. While waiting for the next animal to sample, R recorded the eartag number that corresponded to this kill number from the cattle passport on the recording sheet.

Faecal sample collection

R observed cattle until an animal with a tag was seen arriving towards the skinning point. When the animal arrived in front of R he/she recorded the eartag number next to the kill number on the recording sheet. At this stage the skin is removed and therefore the tag was lost. The AE based opposite the skinning point was responsible for placing a plastic tag on the carcass to ensure correct identification of the animal further on. The animal then arrived at the AE who is responsible for evisceration and removing the gall bladder. The plastic tag reminded the AE not to remove the gall bladder of the tagged animal. R made sure this happened or collected the gall bladder as it reached

the belt where intestines were carried. When the intestinal tract arrived at the belt, R identified the colon, cut through it using scissors and collected a faecal sample into a pre-labelled (kill number and date) universal tube. Scissors were washed at the nearest disinfectant station and gloves changed between samplings.

Liver and gall bladder collection

As described above, the offal remover did not remove gall bladders from tagged animals. If gall bladder was accidentally dropped at the evisceration point, a plastic tag was placed on the liver by AE. Meat inspectors (MI) assessed the livers with gall bladder/plastic tags on, recorded their decision on the offal rejections recording screen as they do with all inspected offal and let R know whether the liver was rejected or not. MI then removed gall bladder and placed it into plastic bag held by R. Lastly, MI placed the whole liver in the bigger plastic bag held by R. Sample bags were pre-labelled with kill numbers. After collection R placed a second label (kill number) inside the liver and gall bladder bags and tied them. Bags containing livers were then placed in a large box and gall bladders in a small box. When boxes were full, they were transported to the carcass fridge in order to keep them cold during the day. R recorded MI's decision on the recording sheet and prepared for the next sampling.

Upon return to the campus, all boxes containing livers and gall bladders were placed in the R(D)SVS Post Mortem Cold Room. 2g of faecal samples were transferred to 15ml falcon tubes, permanently labelled and stored in the -20 °C freezer. The remaining faecal samples were then stored in the fridge for further processing. Blood tubes were placed in the fridge for 24 hrs before serum extraction. On the next day serum was transferred to permanently labelled nunc and eppendorf tubes and stored at -20 °C.

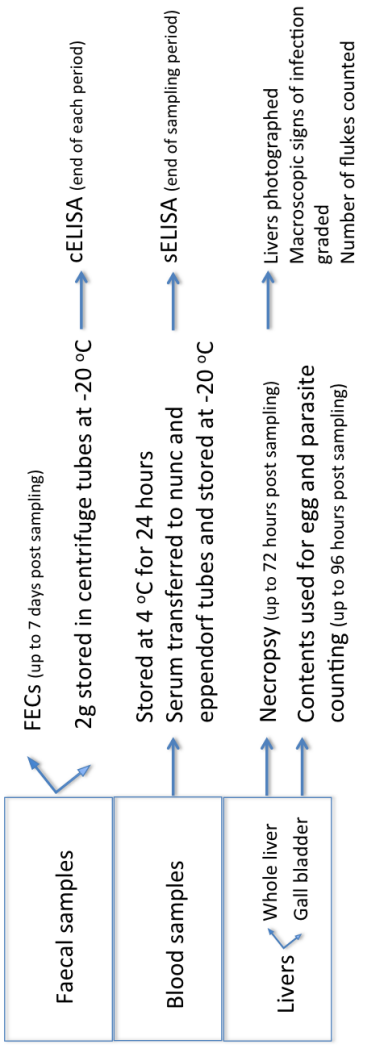


Figure 2.2: Overview of the abattoir based sampling procedure.

Rumen fluke study

During the second and third sampling seasons of the study forestomachs of sampled animals were inspected for the presence of rumen fluke parasites. More precisely, during sampling period "autumn 2014" samples were taken during 5 sampling days between 25th August and 6th October 2014 and during sampling period "winter 2014" samples were taken during 5 sampling days between 13th January and 3rd March 2014. In order to identify the correct forestomach to sample, plastic tags were placed on the forestomach at the time of faecal sampling, labelled with the kill number of that animal. This AEs in the gut room that this forestomach needed to be sampled and it was hence passed on to the researcher for further processing. Forestomachs were incised along the greater curvature of the rumen and everted to remove their contents, as a standard part of the abattoir's tripe preparation process. Everted tagged forestomachs were arranged in a predetermined manner in dorso-medial and ventro-lateral planes as shown in Figure 2.3 for enumeration and mapping of the distribution of rumen flukes. Between 1 and 100 rumen flukes from each parasitised animal, depending on the numbers of flukes present, were fixed in 70% ethanol and archived for future studies. This procedure was carried out as part of a separate study (140), but results will be used here to assess the copro-antigen and the serum-antibody ELISAs for cross-reactivity with rumen fluke as well as report the proportion of animals co-infected with both liver and rumen fluke.

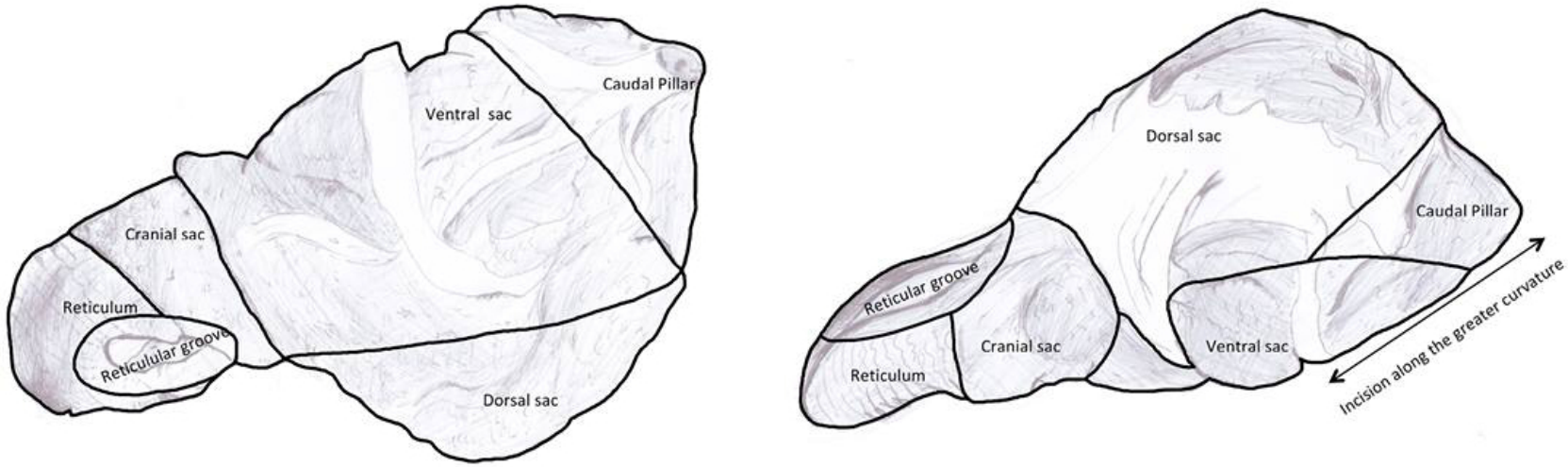


Figure 2.3: Schematic representations of everted tagged forestomachs. Forestomachs were viewed in dorso-medial (left) and ventro-lateral (right) planes and the schematics were used for enumeration and mapping of the distribution of rumen flukes.

2.5 Diagnostic tests

2.5.1 Necropsy

a) Liver dissection Livers were laid out on a tray and the plastic bag containing the liver was rinsed out into the bucket corresponding to the liver to make sure no liver flukes were left in the bag. Fresh incisions parallel to and approximately 1 cm apart from the meat inspector's incisions were made. Fibrosis scores from 0 to 3 (no, mild, moderate, severe) were assigned; 0 - no signs of fibrosis, 1 - mild focal fibrosis, 2 - severe local fibrosis or mild generalised fibrosis, 3 - severe local fibrosis with calcified biled ducts or severe generalised fibrosis. Fibrosis scores were assigned before slicing the liver further in order to mimic what a meat inspector would be able to see on the offal line in the abattoir. Figure 2.4 shows photographic examples of each fibrosis score. The liver was cut into 1-2 cm slices thick and each slice was squeezed in order to collect flukes present. The slices were then placed in a bucket containing lukewarm water for approximately 30 minutes. Water contents were then poured through a 200μm sieve and inspected to retrieve flukes. Each slice was squeezed so that fluke exited the bile ducts, rinsed with water flowing in the bucket and discarded. Water remaining in the bucket was poured through the 200μm sieve and inspected to retrieve remaining flukes. Any fluke recovered at any stage of the procedure was placed in plastic bowl corresponding to the liver. Flukes were then counted and stored in formalin. The total number of flukes was based on the number of whole flukes plus the number of anterior or posterior fluke parts depending on which one was greater (27; 141).

b) Gall bladder egg count A 2-3 cm incision was made at the tip of the gall bladder and gall bladder contents were sieved through a series of 250 and 150μm sieves and collected in a measuring flask. Any contents of the bag containing the gall blad-

der were also sieved through to ensure no eggs were missed and the bag was rinsed out into the sieves for the same reason. Contents were collected into a 500 ml jar and allowed to sediment for 3 minutes. Excess liquid was removed and the remaining liquid was agitated and poured into a narrow bottomed glass. Water was added to the jar and poured into the glass to ensure no eggs remained in the flask. Glass contents were allowed to sediment for 3 minutes. Excess water was then removed with a syringe and liquid was poured in a 15ml falcon tube and allowed to sediment for another 3 minutes. Excess water was syphoned off and the sediment was collected in a petri dish for counting. One drop of 0.5% methylene blue was added and all the eggs on the plate were counted using a stereoscopic dissecting microscope (86). If too many eggs were present making it impossible to count manually (subjective decision based on the author's ability to count the eggs), the contents of the petri dish were diluted before counting. This was done by transferring the contents to a clean 50 ml falcon tube, mixing well and taking a 2ml sub-sample. The eggs in the sub-sample were counted and then multiplied by 25 to estimate the total number of eggs in the sample.

An animal was classified as positive for liver necropsy when 1 or more parasites was found in the liver and/or 1 or more eggs were found in the gall bladder.

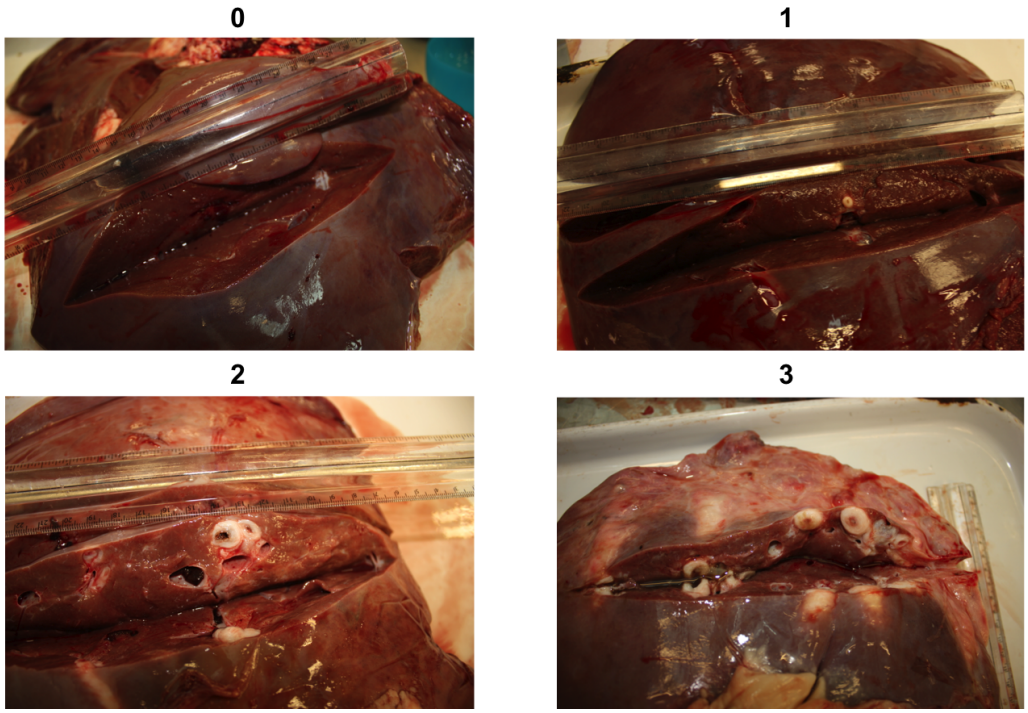


Figure 2.4: Photographic examples of fibrosis scoring. 0 - no signs of fibrosis, 1 - mild focal fibrosis, 2 - severe local fibrosis or mild generalised fibrosis, 3 - severe local fibrosis with calcified biled ducts or severe generalised fibrosis.

2.5.2 Faecal egg count (FEC)

The faecal sample was mixed using a spatula and 5g were weighed out in a measuring cylinder. Water was added up to the 40ml mark and contents were mixed using a stirring rod. Contents were sieved through a coffee strainer and collected in a 250ml beaker for removal of coarse faecal material. Extra water was added to the measuring cylinder and poured through the coffee strainer to make sure all eggs were transferred. The contents were then sieved through a 150μm sieve, collected into a narrow bottomed glass and allowed to sediment for 3 minutes. Extra water was added to the beaker and poured through the sieve to make sure all eggs are transferred. Excess liquid was syringed off and sediment was transferred into a 15ml falcon tube and allowed to sediment for 3 minutes. Excess liquid was syphoned off, making sure the sediment was not disturbed and the sediment was then transferred onto a petri dish. One drop of 0.5 % methylene blue was added and all the eggs on the plate were counted using a stereoscopic dissecting microscope (71). The number of both liver fluke (golden) and rumen fluke (clear) eggs was counted using a click counter. If too many eggs were present making it impossible to count manually, the contents of the petri dish were diluted before counting the number of eggs in a sub-sample as described above. A sample was classified as positive when 1 or more eggs were found in the sample.

2.5.3 BIO K201 Fasciola Coproantigen ELISA (cELISA)

Faecal samples were tested for the presence of excretory-secretory antigens using the commercially available *Fasciola hepatica* antigen ELISA kit (Bio-X Diagnostics, Belgium). The test was performed following the manufacturer's instructions (83) and results were expressed as the sample optical density (OD) as a percentage of the mean positive control OD.

$$\text{Percent positive} = \frac{\text{Sample OD}}{\text{Mean positive control OD}} * 100$$

Samples were classified as positive or negative according to the cut-offs provided by the manufacturer for each batch. The detailed procedure is described below.

Sample preparation Samples and dilution buffer were left on the bench for one hour to reach room temperature. Samples were placed on tissue paper to thaw. Kit dilution buffer was poured in a 500ml sterile bottle. Distilled water was poured into the dilution buffer bottle and emptied into the 500ml bottle a few times. Distilled water was topped up to 250 ml for a 50 ml buffer. 2 ml buffer was added to each sample and samples were vortexed so that no material remained stuck on tube wall. Samples were then centrifuged at 22 rpm for 10 mins. The lid of each eppendorf was opened carefully to avoid mixing sediment with supernatant and the supernatant was then transferred to a pre-labelled eppendorf tube using a pasteur pipette. Samples were placed in the -20 °C freezer until processing.

The cELISA procedure Samples were placed on tissue paper in the same order as they were placed on the ELISA plate for 1 hour to reach room temperature. The positive control, dilution buffer and washing solution were also taken out at the same time. Positive control was reconstituted with 0.5 ml of distilled water and left to dissolve.

Washing solution was diluted with distilled water (x20). Distilled water was poured into wash solution bottle a few times to make sure all crystals were out. The plate strips were labelled on either end as shown in Figure 2.5 in case they came out during washing. 100-μl aliquots of diluted samples and controls were added to the wells as follows. Positive control was added in wells A1 and B1, sample 1 in wells C1 and D1, sample 4 in wells A2 and B2 and so on. When all samples were placed, the ELISA plate was wrapped with cling film and incubated at room temperature on a plate agitator for 2 hours. The biotin-linked anti-*Fasciola hepatica* conjugate was taken out of the fridge one hour before the end of the incubation time in order to reach room temperature. The conjugate was then diluted (x 50) with dilution buffer in a 50 ml falcon tube shaken gently to mix avoiding bubble formation and then poured in the appropriate container for pipetting into the plates. When the 2 hour incubation was completed the plates were washed with washing solution using the following procedure. The plate was emptied of its contents by flipping it sharply over a sink. The plate was then tapped upside down against a piece of absorbent paper until all the liquid was removed. All wells were then filled with washing solution using a spray bottle, emptied over a sink and tapped against absorbent paper as described before. This was repeated two more times. Any remaining bubbles were removed by carefully bursting them using a different pipette tip per well. When the plate was ready 100 μl of conjugate solution was added to each well. The plate was wrapped with cling film and incubated at room temperature for one hour. The avidine-peroxidase conjugate was taken out of the fridge one hour before the end of incubation time in order to reach room temperature. The conjugate was then diluted (x 50) with dilution buffer in a 50 ml falcon tube shaken gently to mix avoiding bubble formation and then poured in the appropriate container for pipetting into plates. When the 1 hour incubation was completed the plates were washed as described before and 100 μl of the diluted peroxidase-linked conjugate solution was added to each well. The plates were then wrapped with cling film and incubated at room temperature

for one hour. When incubation was completed plates were washed as before. 100 μl of chromogen solution was added to each well and the plates were incubated for 10 minutes away from light (in a drawer). At the end of the 10 minutes 50 μl of stop solution per well was added. The optical densities in the wells were read at 450nm using an automated microplate reader (Thermo Scientific Multiskan Go). Each plate was validated if the difference yielded by the positive control antigen was greater than the validation value provided in the manufacturer's protocol. The ELISA kits used for sampling period 1 and 2 came from the same batch, so the validation cut-off was $>$ 1.001, while a new batch was used for the last sampling period for which the validation cut-off was 0.800. Similarly, the cut-off for a positive result was 7.49 % and 7.00 % for each batch respectively.

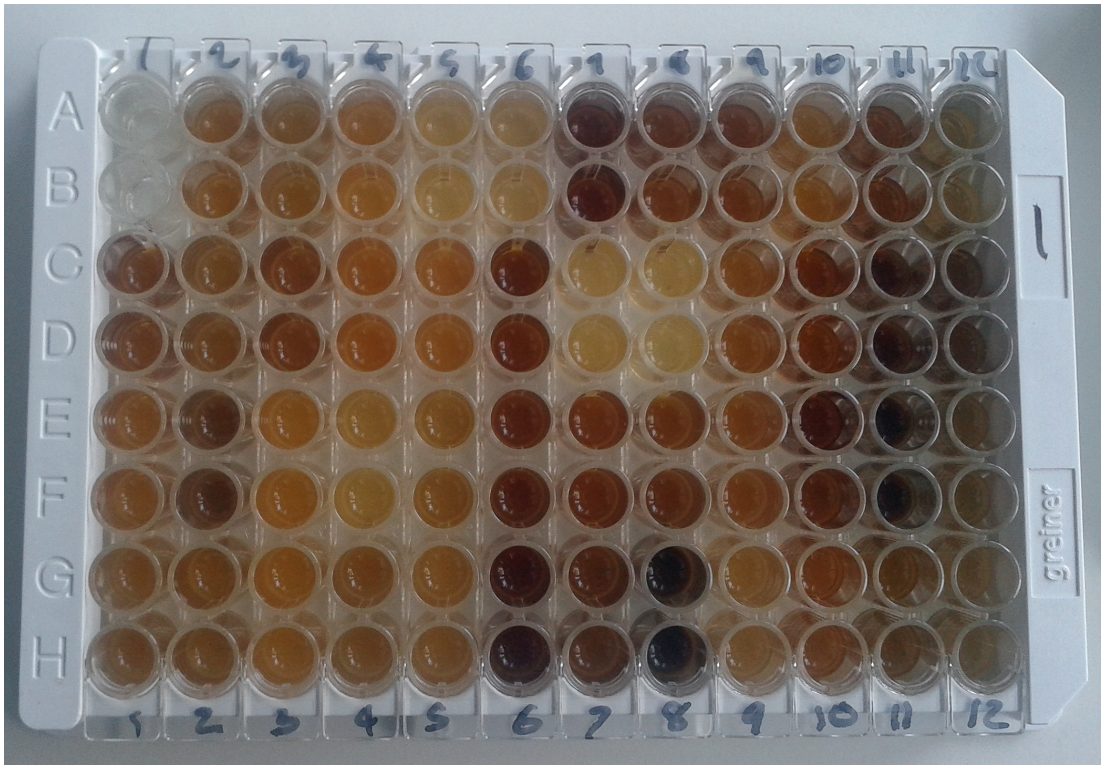


Figure 2.5: An example of a cELISA plate. Plate strips were labelled on either end as shown in case they came out during washing.

2.5.4 Serum antibody ELISA (sELISA)

Serum samples were analysed using the excretory/secretory (ES) antibody ELISA developed by the Liverpool School of Tropical Medicine (142). The procedure described by Salimi-Bejestani et al. (2005) (142) was performed with the following modifications:

- 1:8000 monoclonal mouse anti-bovine IgG conjugate (AbD Serotec, Bio-Rad Laboratories Inc, Hertfordshire, UK) was used
- A new positive control was used so the equation used for calculating the results was slightly varied to obtain comparable results to previous controls. The percent positive (PP) value was obtained by the quotient of the mean sample OD (based on two duplicates) divided by the mean positive control OD (four duplicates), which was then multiplied by 111 instead of 100 to account for the new positive control as suggested by the test developers at Liverpool (Prof. D. Williams, 2014, pers.comm., 1 Dec).

$$\text{Percent positive} = \frac{\text{Mean test sample OD}}{\text{Mean positive control OD}} * 111$$

Samples were classified as positive if they had a PP greater or equal to 10. The controls (++ = high, + = low, - = negative) and *F. hepatica* antigen were provided by the Department of Veterinary Parasitology, University of Liverpool, Liverpool UK, where the author was trained on performing this in house ELISA. Sample testing and identification of a suitable conjugate concentration using a checkerboard were carried out in R(D)SVS, Edinburgh, UK. The detailed procedure is described below.

Checkerboard As a new conjugate was used, a checkerboard was used to determine the conjugate dilution that gave the high positive control an OD of around 1.0 – 1.2

and the negative control an OD of around 0.05 (Prof. D. Williams, 2014, pers.comm., 4 Dec). Keeping the concentrations of the serum controls and the antigen constant, the ELISA was run using a series of different conjugate dilutions (1:4000, 1:8000, 1:12400, 1:17000, 1:22400, 1:27500). The checkerboard plate outline and results are shown in Figure 2.6a. According to the criteria shown above dilution 1:8000 was chosen.

Reagent Preparation PBS was prepared by mixing 90g of NaCl, 18.5g of NA_2HPO_4 anhydrous and 5.375g of KH_2PO_4 in 2.5L distilled water. This was diluted (1:4) for use. The diluted PBS was used to prepare the wash buffer (pH 7.2 PBS containing 0.05% Tween-20 (PBS-Tween)). The blocking buffer (BF) was prepared mixing 2g of skimmed milk powder (Marvel, Premier International Foods®, Spalding UK) with 100ml wash buffer (WB) gently to avoid bubble formation. Lastly, the coating buffer (CB) was prepared by adding 0.159 Na_2CO_3 anhydrous and 0.292g $NaHCO_3$ to 100ml of distilled water, mixing and regulating pH to 9.6 using HCl.

Plate coating The plate preparation was carried out the evening before an ELISA was run. The ES antigen was diluted in CB at a 1/354 dilution. The Immulon-2 ELISA 96-well plates (Fisher Scientific) were coated with 100 μl of diluted antigen, covered with adhesive plate sealer and incubated at room temperature for 1 hour. The plate was then refrigerated overnight.

The sELISA procedure Plates, samples, controls, TMB substrate (MAST Diagnostics, Bootle, Merseyside, UK) and BF were taken out of the fridge to reach room temperature. The plates were washed using the following procedure. Contents were emptied by flipping plates sharply over a sink. Wash buffer was added to all wells and plates were emptied again. This was repeated twice. Wash buffer was added again

and plates were left to soak for 5 minutes before emptying. The whole procedure was repeated one more time. The plate was then tapped upside down against a piece of absorbent paper until all the liquid was removed. Any remaining bubbles were removed by carefully bursting them using a different pipette tip per well. After washing, 200 μl of BF were added to each well, the plate was covered with adhesive plate sealer and incubated at 37 °C for 1 hour. At the end of the incubation period the plate was washed as above. 1:800 dilutions of samples and controls were then added to the plate following the outline shown in Figure 2.6b. Only BB was added to cells G1, G2, H1 and H2. The plate was then incubated at 37 °C for 1 hour and washed as described before. 100 μl of 1:8000 monoclonal mouse anti-bovine IgG conjugate (AbD Serotec, Bio-Rad Laboratories Inc, Hertfordshire, UK) was added to all wells except H1 and H2 where 100 μl of BF was added. The plate was then incubated at 37 °C for 1 hour and washed as described before. After that, 100 μl of TMB was added to each well, the plate was covered and incubated at room temperature (in a drawer) for 20 minutes. Lastly, 100 μl stopping solution (0.5M HCl 25ml + 475ml distilled water) was added to each well and the plate was read at 450nm using an automated microplate reader (Thermo Scientific Multiskan Go).

	1	2	3	4	5	6	7	8	9	10	11	12
A	++	++	S1	S1								
B	++	++	S2	S2								
C	+	+										
D	+	+										
E	-	-										
F	-	-										
G	CB	CB									S39	S39
H	BB	BB	S8	S8							S40	S40

(a)

	CONJUGATE DILUTION											
	1:4000		1:8000		1:12400		1:17000		1:22400		1:27500	
++	2.5645	2.1915	1.341	1.182	0.841	0.78	0.5875	0.6075	0.436	0.404	0.3245	0.2625
	2.5475	2.3965	1.387	1.192	0.801	0.714	0.5725	0.4725	0.385	0.395	0.2865	0.2075
+	1.5405	1.4385	0.692	0.619	0.417	0.385	0.3045	0.2555	0.176	0.219	0.1375	0.1065
	1.5425	1.5125	0.739	0.673	0.438	0.413	0.3145	0.2705	0.195	0.24	0.1405	0.1205
-	0.0505	0.0515	0.029	0.036	0.013	0.004	0.0095	0.0185	0.018	0.018	0.0025	-0.0055
	0.0565	0.0575	0.038	0.027	0.016	0.008	0.0085	0.0095	0.013	0.01	-0.0025	0.0015
CB	0.034	0.0185	0.01	0.015	0.007	0.002	0.0035	0.0035	0.004	0.01	-0.0015	0.0005
BB	-	-	-	-	-	-	-	-	-	-	-	-

(b)

Figure 2.6: sELISA plates a) Checkerboard results b) sELISA plate outline. Key: ++ = high positive control, + = low positive control, - = negative control, CB = conjugate buffer, BB = blocking buffer.

Chapter 3

Evaluation of the performance of five diagnostic tests for Fasciola hepatica infection in naturally infected cattle using a Bayesian no gold standard approach.

3.1 Abstract

The clinical and economic importance of fasciolosis has been recognised for centuries, yet diagnostic tests available for cattle are far from perfect. Test evaluation has mainly been carried out using gold standard approaches or under experimental settings, the limitations of which are well known. In this study, a Bayesian no gold standard approach was used to estimate the diagnostic sensitivity and specificity of five tests for fasciolosis in cattle. These included detailed liver necropsy including gall bladder egg

counts, faecal egg counting, a commercially available copro-antigen ELISA, an in-house serum excretory/secretory antibody ELISA and routine abattoir liver inspection. In total 619 cattle slaughtered at one of Scotland's biggest abattoir were sampled, during three sampling periods spanning summer 2013, winter 2014 and autumn 2014. Test sensitivities and specificities were estimated using an extension of the Hui Walter no gold standard model, where estimates were allowed to vary between seasons if tests were *a priori* believed to perform differently for any reason. The results of this analysis provide novel information on the performance of these tests in a naturally infected cattle population and at different times of the year when different prevalences of acute or chronic infection are expected. Accurate estimates of sensitivity and specificity will allow for routine abattoir liver inspection to be used as a tool for monitoring the epidemiology of *F. hepatica* as well as evaluating herd health planning. Furthermore, the results provide evidence to suggest that the copro-antigen ELISA does not cross-react with *Calicophoron daubneyi* rumen fluke parasites, while the serum antibody ELISA does.

3.2 Introduction

Fasciolosis, first reported in 1379, has been recognised as a clinically and economically important disease for centuries (4). The infection caused by trematode parasites of the genus *Fasciola* can infect many mammals including sheep, cattle, goats, deer and humans (5). In cattle, fasciolosis mainly manifests in its chronic form, which can lead to weight loss, anaemia and hypoproteinaemia. Clinical signs are often mild and may present as loss of productivity, while in severe cases sub-mandibular oedema may be seen. Unlike sheep, cattle liver pathology includes bile duct calcification and gall-bladder enlargement (5; 143). Globally, the infection is estimated to cost the livestock industry €2.5 billion per year (144), while losses due to liver fluke have been estimated

to range between €1100-2000 million per year in the European Union (145).

In the UK and other temperate regions, *F. hepatica* is the most common aetiological agent of fasciolosis (5). *F. hepatica* has a complicated multi-host, highly climate dependent life cycle which takes typically between 18 and 30 weeks to be completed. The mud snail, *Galba truncatula* is the most common intermediate host of *F. hepatica* in Europe (143; 11). Temperature and moisture levels play an important role in the parasite's life cycle and it is generally accepted that average daily temperatures of more than 10 ^{o}C and high moisture levels are required for both the egg development and the reproduction of the parasite within the snail (15). This results in seasonal increases of the incidence of infection, which vary between years depending heavily on climatic conditions.

The incidence of fasciolosis in the UK has been reported to have increased during the last decade and more importantly its distribution has changed. In the past, fasciolosis was most commonly seen in the wetter western regions of the country, while it is now evident that the disease has become endemic in the previously drier eastern regions (7; 29). Reasons for the changing epidemiology of *F. hepatica* are thought to include climate change, increasing animal movements and development of triclabendazole resistance (31). Unpredictable weather conditions and resistance to anthelmintic treatment make control strategies less straightforward to plan. This increases the need for appropriate use of diagnostic tests, which along with improved knowledge and consideration of their limitations, can enhance implementation of more effective management strategies.

The development of tests for the correct diagnosis of the infection has been going on for years, yet no test developed so far has been shown to have adequately high sensitivity and specificity in the field setting. Research on performance of available diagnostic tests in cattle, and especially the copro-antigen ELISA, is far from complete.

The faecal egg count test, is commonly used in practice but can only detect patent infections. The serum antibody ELISA has the limitation of providing information on exposure rather than current infection but can detect exposure even at pre-patent stages of infection (142). On the other hand the copro-antigen ELISA, which detects *F. hepatica* excretory-secretory antigens in faeces, is reported to detect early stages of infection without the limitation of giving positive results due to past exposure (83; 86). This test has been evaluated by different research groups with varying results in sheep, but little has been reported on its performance in cattle (85).

Furthermore, inspection of livers of cattle slaughtered in abattoirs across Europe for signs of liver fluke is mandatory according to Regulation (EC) No 854/2004. In a previous study in Switzerland, Rapsch et al. (75) estimated the sensitivity of abattoir liver inspection to be 63.2%. Such estimates are expected to vary between countries, hence it is important to be able to obtain estimates specific to each country. Lastly, detailed liver necropsy techniques including gall bladder egg counts are available for research purposes, but impractical and expensive for routine use. These are expected to be extremely sensitive, even though there is still a window of error in case of very early stage infections. Moreover they can provide information on the severity of infection according to the degree of damage, as well as the fluke burden.

In this study we have used the above diagnostic tests on samples taken from Scotbeef, Scotland's largest red meat abattoir, receiving animals from all around Scotland, northern England and Northern Ireland in an attempt to improve our knowledge on the performance of these diagnostic tests in the UK setting. More precisely this analysis aims to estimate: i) the performance of meat inspection as a tool for diagnosis of *F. hepatica* infection; and ii) the performance of liver necropsy, serum antibody ELISA, the copro-antigen ELISA and faecal egg count diagnostic tests.

3.3 Materials and methods

3.3.1 Abattoir Based Sampling

Samples, including blood, faeces, whole livers and gall bladders from each animal, were collected during the three sampling periods; “summer 2013“ , “winter 2014“ and “autumn 2014“. A detailed description of the sampling strategy can be found in chapter 2.

3.3.2 Diagnostic Tests

Every animal sampled was tested using five diagnostic diagnostic tests. These included liver necropsy, faecal egg counting (FEC), a copro antigen ELISA (cELISA), a serum antibody ELISA (sELISA) and liver inspection. The detailed protocol followed for each test is described in chapter 2. In terms of liver necropsy, an animal was classified as positive for liver necropsy when 1 or more parasites was found in the liver and/or 1 or more eggs were found in the gall bladder. In terms of FEC, animals were classified as positive when 1 or more eggs were found in the sample. For the cELISA, samples were classified as positive or negative according to the cut-offs provided by the manufacturer for each batch. Furthermore, in terms of the sELISA, animals were classified as positive if they had a PP greater or equal to 10. Finally, for the purposes of this analysis, in terms of liver inspection carried out at the abattoir by the Meat Hygiene Service (MHS), animals were considered positive if their liver inspection MHS results were “Active” and “Historic”, and negative if they were “No fluke”.

In terms of rumen fluke, the number of rumen fluke eggs per gram was recorded for every faecal sample collected and the number of rumen fluke parasites found in the forestomach was recorded during “winter 2014“ and “autumn 2014“ sampling periods.

The exact methodology is described in chapter 2.

3.3.3 Statistical Analysis

3.3.4 A. The No Gold Standard (NGS) estimation of diagnostic test performance

NGS, introduced by Hui & Walter (146), is a latent class approach to the evaluation of diagnostic tests when a "gold standard" is not available. The Bayesian version incorporates prior knowledge by specifying prior distributions for test properties and prevalence. If no prior information is available, vague, uniform priors are set. Probabilities of all the possible combinations of test outcomes conditional on the unknown disease status are specified using the sensitivity (Se) and specificity (Sp) of each test and the prevalence (p) of each sub-population, in this case periods "summer 2013", "winter 2014" and "autumn 2014" (147; 75). Animals can be positive or negative for each of the five tests included in this analysis so there are 2^5 (i.e. 32) possible combinations of test results. Hence, for each sub-population the counts of animals (O_i) of each combination of test results, in this case 32 (S) combinations for the five tests (T), follow a multinomial distribution (148; 149):

$$O_i|Se_j, Sp_j, p_i \sim \text{Multinomial}(Pr_i, n_i) \text{ for i=1,2,...,S and j=1,2,...,T}$$

where Pr_i is the probability of observing the *i*th combination of test results.

Examples of how to specify two such probabilities are shown below:

1. Probability of obtaining a positive result in all five tests

   $\Pr(T_1+, T_2+, T_3+, T_4+, T_5+) =$

   $Se_1Se_2Se_3Se_4Se_5p_i + (1-Sp_1)(1-Sp_2)(1-Sp_3)(1-Sp_4)(1-Sp_5)(1-p_i)$

2. Probability of obtaining a positive result in the first four tests and a negative result in the fifth test

$$\Pr(T_1+, T_2+, T_3+, T_4+, T_5-) =$$
$$\mathrm{Se_1Se_2Se_3Se_4}(1-\mathrm{Se_5})\mathrm{p}_i + (1-\mathrm{Sp_1})(1-\mathrm{Sp_2})(1-\mathrm{Sp_3})(1-\mathrm{Sp_4})\mathrm{Sp_5}(1-\mathrm{p}_i)$$

The ratio of acute versus chronic infection is expected to be different, according to the known lifecycle of the parasite, between the three different times of the year which may affect the sensitivities and/or specificities of certain tests. Therefore, different estimates for the sensitivities of FEC, the copro-antigen and the serum antibody ELISA tests were obtained for each season as well as the specificity of the serum antibody ELISA. This was done for two reasons. Firstly, as shown by Toft et al. (148), if estimates vary between sub-populations the combined estimate will be biased towards the estimate supported by most data i.e. the one from the sub-population with the highest prevalence. Secondly, this can provide information on which tests are more appropriate at different times of the year.

Model Assumptions

1. Tests are conditionally independent. In other words, the misclassification errors of each test are unrelated conditional on the true disease status of the animal. For example, the probability of a truly diseased animal testing positive in test 2 (sensitivity), is not altered by the result of test 1 (150; 151). There are various models for accounting for conditional dependence. In this case we have used the model suggested by Vacek (1985) as described below (150; 148). Ten models including covariance terms (γ_{Se} and γ_{Sp}) for one combination of two tests at a time were specified in order to inspect the effect of adjusting for covariance for each test combination on the sensitivity and specificity estimates of all tests. For example:

a) Probability of obtaining a positive result in all five tests accounting for covariance between tests 1 and 2.

$$\Pr(T_1+, T_2+, T_3+, T_4+, T_5+) =$$
$$(\mathrm{Se}_1\mathrm{Se}_2+\gamma_{\mathrm{Se}})\mathrm{Se}_3\mathrm{Se}_4\mathrm{Se}_5\mathrm{p}_i +$$
$$((1-\mathrm{Sp}_1)(1-\mathrm{Sp}_2)+\gamma_{\mathrm{Sp}})(1-\mathrm{Sp}_3)(1-\mathrm{Sp}_4)(1-\mathrm{Sp}_5)(1-\mathrm{p}_i)$$

b) Probability of obtaining a negative result in the first test and a positive result in all other tests accounting for covariance between tests 1 and 2.

$$\Pr(T_1-, T_2+, T_3+, T_4+, T_5+) =$$
$$((1-\mathrm{Se}_1)\mathrm{Se}_2-\gamma_{\mathrm{Se}})\mathrm{Se}_3\mathrm{Se}_4\mathrm{Se}_5\mathrm{p}_i +$$
$$(Sp_1(1-Sp_2)-\gamma_{\mathrm{Sp}})(1-\mathrm{Sp}_3)(1-\mathrm{Sp}_4)(1-\mathrm{Sp}_5)(1-\mathrm{p}_i)$$

c) Probability of obtaining a negative result in the first two tests and a positive result in all other tests accounting for covariance between tests 1 and 2.

$$\Pr(T_1+, T_2+, T_3+, T_4+, T_5+) =$$
$$((1-\mathrm{Se}_1)(1-\mathrm{Se}_2)+\gamma_{\mathrm{Se}})\mathrm{Se}_3\mathrm{Se}_4\mathrm{Se}_5\mathrm{p}_i +$$
$$(\mathrm{Sp}_1\mathrm{Sp}_2+\gamma_{\mathrm{Sp}})(1-\mathrm{Sp}_3)(1-\mathrm{Sp}_4)(1-\mathrm{Sp}_5)(1-\mathrm{p}_i)$$

2. Test sensitivities and specificities are constant between populations.

3. Prevalences vary between populations.

The original Hui & Walter model contained two tests and two populations. Assumptions 2 and 3 were there to ensure that there are enough degrees of freedom to ensure the model's identifiability. As liver fluke infection levels vary throughout the year and between years, we were able to assume that the prevalence will vary between the three sampling seasons. Additionally, according to Toft et al. when three or more tests are compared one population is enough (148) to have sufficient degrees of freedom. As this model is an adaptation of the original model, with three sub populations and five tests, we ensure that we have enough degrees of freedom to be able to allow the stated sensitivities and specificities to vary between sub-populations and to include covari-

ance terms for one combination of tests at a time.

Model evaluation and identification of a suitable samples size using simulated data

In order to determine whether the proposed methodology can reclaim test parameters using a feasible sample size three sub-populations of animals were simulated, representing the three sampling periods, under a range of plausible diagnostic test parameters and the accuracy of the statistical method in recovering the set parameters over a range of sample sizes was evaluated.

MCMC diagnostics

Markov chain Monte Carlo (MCMC) chain convergence was assessed by visual inspection of the three sample chains using trace and Gelman-Rubin diagnostic plots for each variable in the model (152). A correlation matrix of each chain was plotted to check for high correlation between variables.

Priors

As a Bayesian framework is used in this analysis, prior distributions were specified for the prevalence of each sub-population and the sensitivities and specificities of each test. Vague, uniform priors with an interval between 0 and 1 were used for the prevalence of each sub-population.

$$\mathrm{p} \sim dbeta(1, 1)$$

Similarly for the sensitivities and most of the specificities of evaluated tests, a wide distribution with an interval between 0 and 1 was used to reflect the fact that there is

scarce knowledge on the performance of most of these tests in a real life scenario.

$$\text{Se} \sim dbeta(2,1)$$
$$\text{Sp} \sim dbeta(2,1)$$

Liver necropsy was the only test where the prior distribution given for the specificity was highly informative. As mentioned before an animal was classified as positive for liver necropsy when either at least one fluke was found in the liver and/or when at least one egg was seen in the bile sample. It is therefore very unlikely that an animal can be wrongly classified as positive as liver flukes are easily identifiable and no other eggs similar to *Fasiola hepatica* eggs are expected to be seen in the bile. In a previous study by Rapsch et al. (75) a similar test was assigned a specificity of 1 for the reasons explained. In order to account for the possibility of egg sequestration in the gall bladder for up to three weeks post treatment (108) we chose the following prior distribution instead.

$$\text{Sp}_{\text{liver necropsy}} \sim dbeta(9,1)$$

The analysis was repeated using priors dbeta(1,1) for the Se and Sp of all tests to assess the effect of priors on the posterior estimates.
Priors for the covariance variables, γ_{Se} and γ_{Sp}, were uniform distributions using the following maximum and minimum limits (153; 147).

$$(\text{Se}_1 - 1)(1 - \text{Se}_2) \leq \gamma_{\text{Se}} \leq min(\text{Se}_1, \text{Se}_2) - \text{Se}_1\text{Se}_2$$
$$(\text{Sp}_1 - 1)(1 - \text{Sp}_2) \leq \gamma_{\text{Sp}} \leq min(\text{Sp}_1, \text{Sp}_2) - \text{Sp}_1\text{Sp}_2$$

Model implementation

The model was implemented in JAGS (154), a software which uses MCMC simulations to construct posterior distributions for the analysis of Bayesian hierarchical models. JAGS was run within R (Version 3.0.3) (155) using the *rjags* package(156). The first

20,000 iterations were discarded as burn-in and the following 20,000 iterations were used to construct the posterior distributions. The model specification is included in Appendix B. R Package *coda* (152) was used to carry out MCMC diagnostics and package *corrplot* (157) was used to visualize the correlation matrix between variables. The results were plotted using *ggplot2* (158). A map showing the distribution of sampled animals was plotted using *ggmap* (159) and the map tiles were sourced from Stamen Design (using data by OpenStreetMap), which are freely available under CC BY 3.0 license.

Positive and Negative Predictive Values

Sensitivity and specificity estimates report diagnostic test validity however positive (PPV) and negative predictive values (NPV) are the appropriate measure for interpreting tests in a specific population. They are the probability that a test positive or negative animal is truly positive or negative respectively. This is more easily interpreted by both farmers and vets, but its value depends on the true prevalence of the disease in the population (160). Based on the Bayes formula (161), presented below, one can estimate the predictive values using estimates for sensitivity (Se), specificity (Sp) and the true population prevalence (p) (162).

$$\text{PPV} = \frac{\text{Se}*p}{(\text{Se}*\text{p})+(1-\text{Sp})*(1-\text{p})} \qquad \text{NPV} = \frac{\text{Sp}*(1-p)}{(\text{Sp}*(1-\text{p}))+(1-\text{Se})*\text{p}}$$

PPVs and NPVs of the MHS liver inspection and FECs were calculated using the Se and Sp estimated by the NGS model over a range of possible prevalences to demonstrate this.

3.4 Results

Descriptive statistics

In total, 619 cattle were sampled, 207 during summer 2013, 204 during winter 2014 and 208 during autumn 2014. Cattle age ranged from 369 to 1121 days old (Figure 3.1) and cattle of a variety of breeds were sampled as shown in Figure 3.2. As Figure 3.3 shows, cattle sampled came from Scotland, northern England and Northern Ireland i.e. the geographical distribution of the general population of cattle slaughtered at the abattoir was well represented. Samples from every animal were tested with the five tests mentioned.

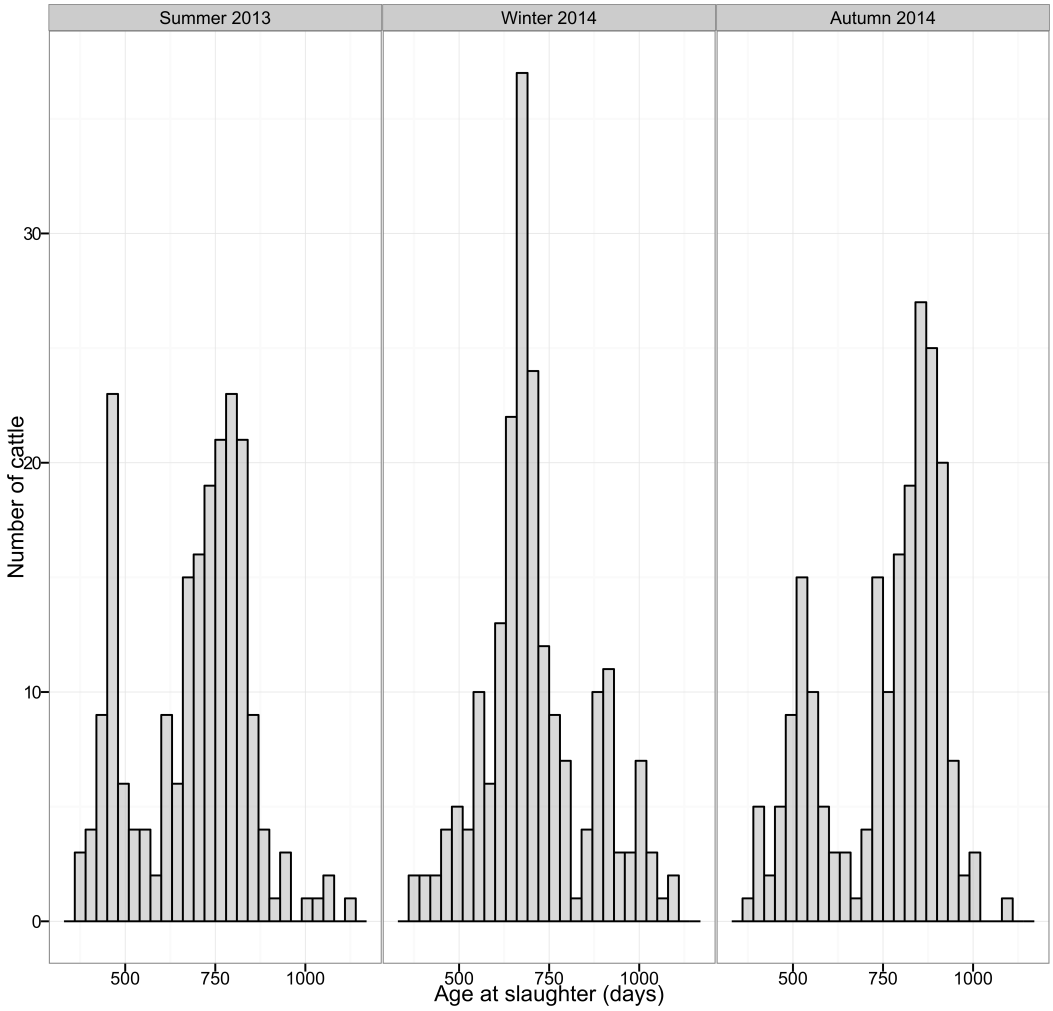


Figure 3.1: Distribution of cattle age per period. The age of cattle sampled ranged from 369 to 1121, with a mean of 720 days old.

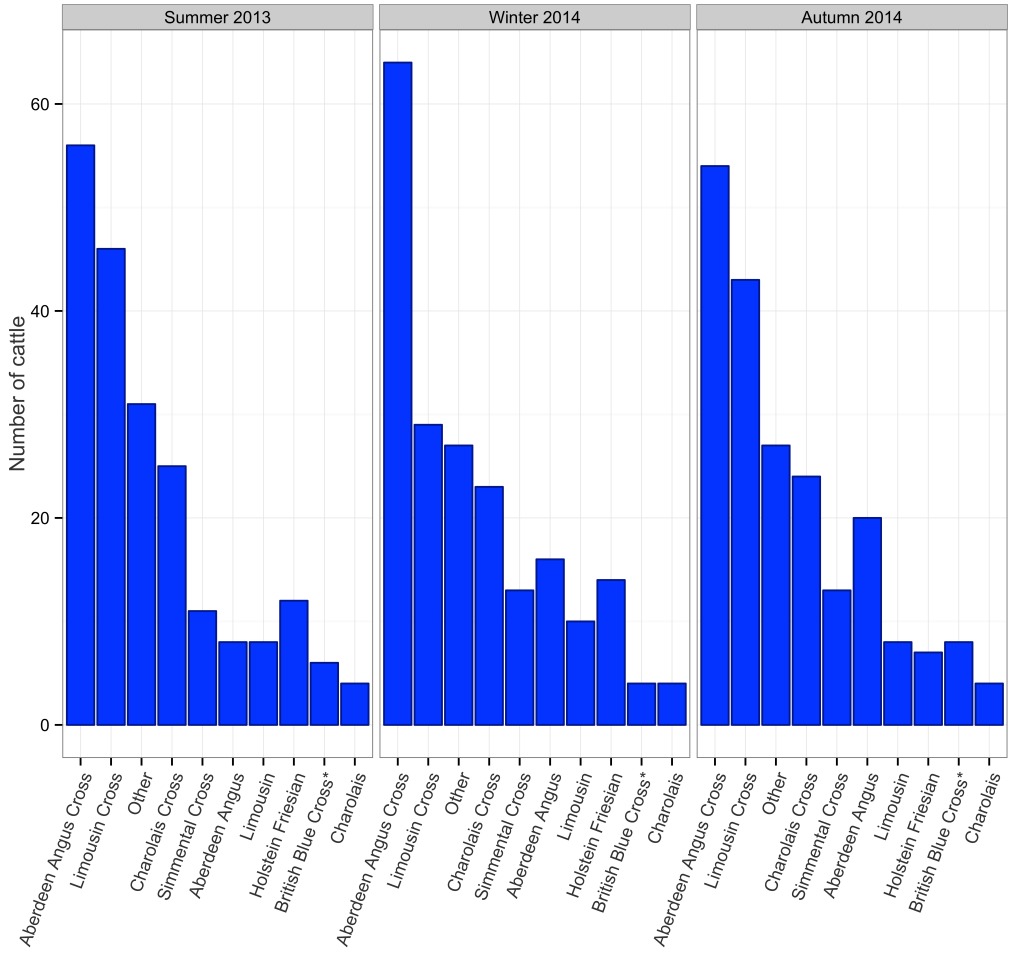


Figure 3.2: Distribution of cattle breed per period. Cattle sampled were of a range of different breeds found in the UK. 175 cattle were Aberdeen Angus cross, 118 were Limousin cross, 73 were Charolais cross, 48 were Aberdeen Angus, 37 were Simmental, 33 Holstein Friesian, 26 Limousin, 18 British Blue cross, 12 Charolais and 85 were of other less common breeds.

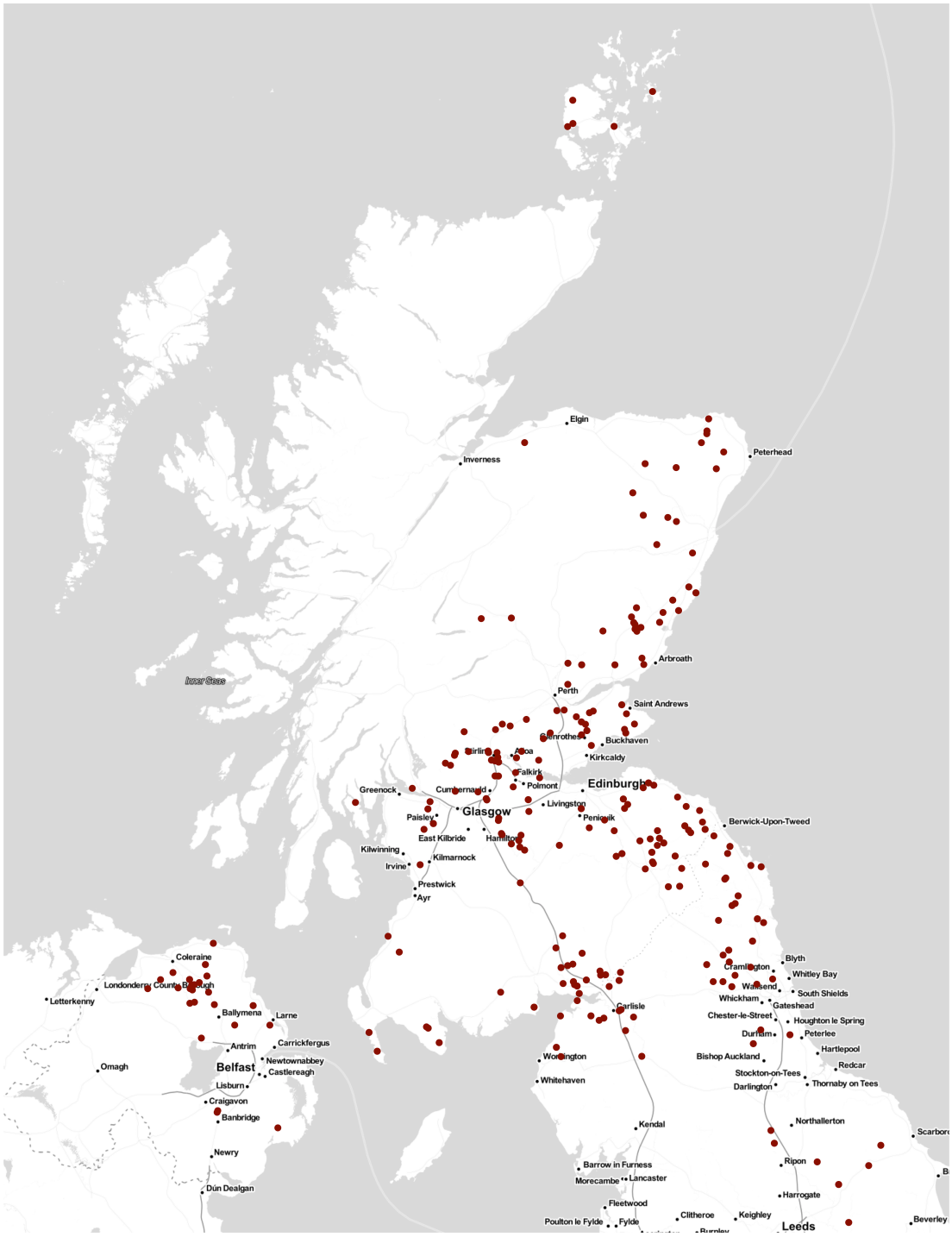


Figure 3.3: Geographical distribution of cattle sampled. Samples used in this study were taken from Scotbeef, one of Scotland's largest red meat abattoirs, receiving animals from all around Scotland, northern England and Northern Ireland. Figure shows the distribution of cattle sampled i.e. the geographical distribution of the general population of cattle slaughtered at the abattoir was well represented. The map was plotted using R package *ggmap* (159) using tiles sourced from Stamen Design (using data by OpenStreetMap).

3.4.1 Diagnostic test results

Table 3.1 shows the binary results of each test per sampling period. Table 3.2 shows the results of liver necropsy according to whether liver flukes were found in the liver and/or whether eggs were found in the gall bladder. Figure 3.4 shows the distribution of parasite burden per fibrosis score as recorded during liver necropsy. Among livers where flukes were found, parasite burden ranged from 1 to 86 parasites, with a mean of 8.5 and a median of 4. Parasite burden reflects the sum of parasites found in the liver and gall bladder. Parasites were mainly retrieved from the livers, except in 5 animals where 1 parasite was also retrieved from the gall bladder. As previously described a fibrosis score was assigned based on a presentation of the liver mimicking the one presented to the MHS. The colour of the points shows the decision taken by the MHS during liver inspection at the abattoir. Higher fibrosis scores appear to have higher parasite burden, but it is also important to note that livers with no signs of fibrosis, that were also not rejected at the abattoir were found to have parasites. Furthermore, many livers which were classified as "Historic" by the MHS (green) were found to have parasites. Lastly, livers classified as "Active" by the MHS (red) appear to be spread evenly among fibrosis scores 1 to 3, while there were a few livers with a fibrosis score 0 which were classified as "Active". This might mean that what was presented at the liver necropsy was not always the same as what was seen by the MHS. Lastly, Table 3.3 shows the descriptive statistics of the continuous results of each diagnostic test per sampling season. Histograms of the distribution of these results are included in Figures B.1 to B.7 in Appendix B.

Table 3.1: Proportions of test positives for each test and number of animals sampled.

	Summer 2013	Winter 2014	Autumn 2014	Overall
Number sampled	207	204	208	619
MHS inspection	0.32	0.29	0.25	0.29
Necropsy	0.39	0.33	0.23	0.32
cELISA	0.29	0.25	0.18	0.24
FEC	0.31	0.25	0.13	0.23
sELISA	0.35	0.36	0.37	0.36

Table 3.2: Liver necropsy detailed results

		Liver Burden >0	
		No	Yes
Gall bladder egg no >0	No	421	5
	Yes	32	158

Table 3.3: Descriptive statistics of the continuous diagnostic test results per sampling season.

Diagnostic test	Min	Median	Mean	Max	NA	Season
Gall bladder	0	0	659.50	19320	2	Summer 2013
eggs no	0	0	1092.00	43320	1	Winter 2014
	0	0	363.70	9200	0	Autumn 2014
Liver fluke	0	0	3.21	86	0	Summer 2013
burden	0	0	2.87	74	0	Winter 2014
	0	0	0.71	23	0	Autumn 2014
Rumen fluke	0	0	84.04	2530	36	Winter 2014
burden	0	0	38.38	1470	37	Autumn 2014
FEC	0	0	2.51	118.00	0	Summer 2013
liver fluke	0	0	0.43	20.80	0	Winter 2014
	0	0	0.12	3.40	0	Autumn 2014
FEC	0	0	3.30	215.20	36	Summer 2013
rumen fluke	0	0	8.50	220.40	0	Winter 2014
	0	0	4.11	192.00	0	Autumn 2014
cELISA	-0.97	0.43	16.08	117.60	0	Summer 2013
	-1.89	0.43	14.29	127.70	0	Winter 2014
	-1.85	0.10	7.52	112.90	0	Autumn 2014
sELISA	0.00	4.50	16.61	121.90	0	Summer 2013
	0.85	5.11	17.36	107.30	0	Winter 2014
	0.48	5.84	19.31	148.70	0	Autumn 2014

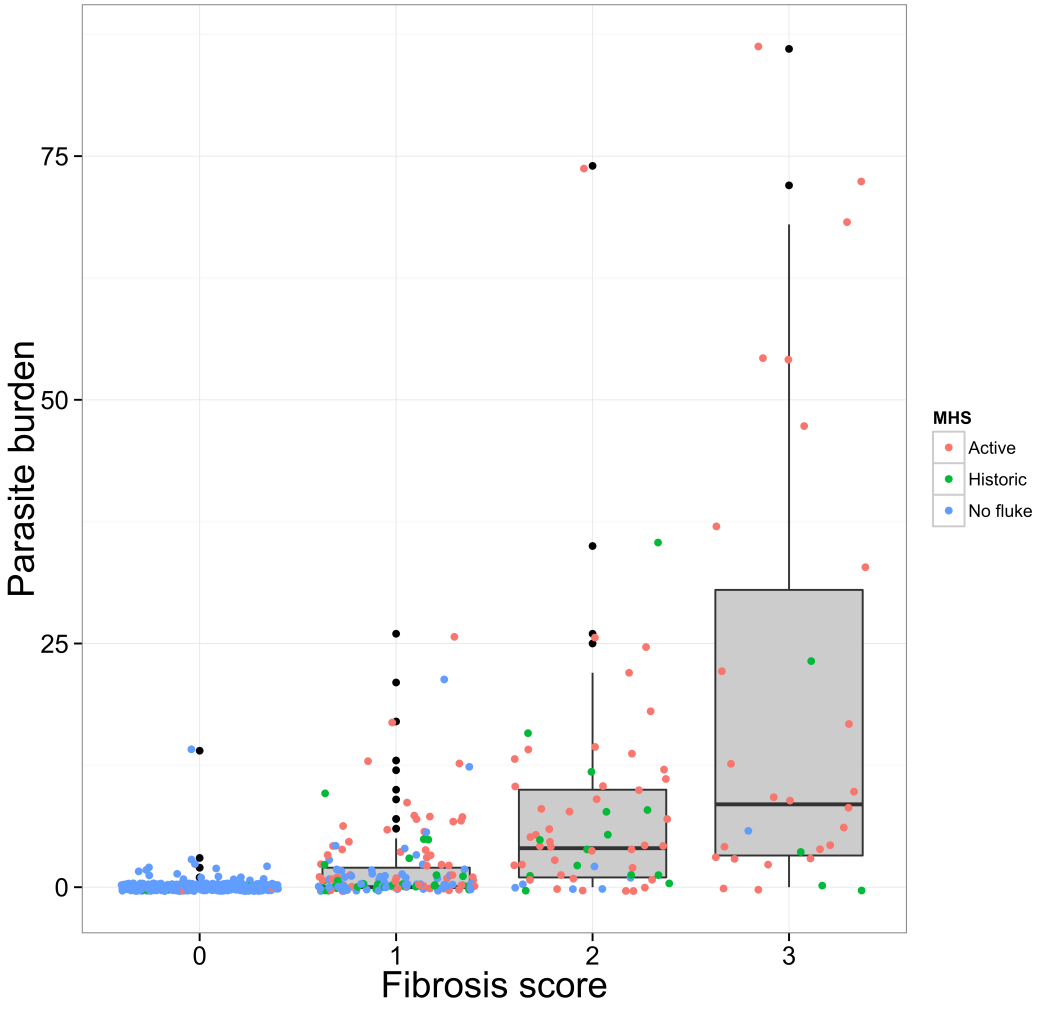


Figure 3.4: Distribution of parasite counts by fibrosis score and MHS classification. Figure shows the distribution of parasite burden per fibrosis score as recorded during liver necropsy. Among livers in which were found, parasite burden ranged from 1 to 86 parasites, with a mean of 8.5 and a median of 4. A fibrosis score was assigned based on a presentation of the liver mimicking the one presented to the MHS. The colour of the points shows the decision taken by the MHS during liver inspection at the abattoir.

3.4.2 Liver fluke and rumen fluke co-infection

Table 3.4 shows the crosstabulation of the binary results of liver necropsy and rumen fluke forestomach burden. While 44 animals were found to be co-infected with both parasites, 51 were only infected with liver fluke and 53 were only infected with rumen fluke.

Table 3.4: Cross tabulation of liver fluke affected animals vs. rumen fluke affected animals.

		Rumen fluke status		
		Positive	Negative	Total
Liver fluke status	Positive	44	51	95
	Negative	53	191	244
	Total	97	242	339

3.4.3 Cross reactivity of ELISA based tests with rumen fluke

Results of both ELISA based test were compared with the results of rumen fluke counts in order to check whether any false positives could be due to cross reaction with rumen fluke parasites. Out of 244 cattle with negative liver necropsy results, 53 were found to have at least one fluke in the rumen (Table 3.4). None of those samples had a positive copro-antigen result suggesting that cELISA does not cross react with rumen fluke in cattle. On the other hand, 18 of those animals had positive sELISA results. While we do not know whether this is due to previous exposure to *F. hepatica*, this may be an indication of cross-reactivity of the sELISA with rumen fluke.

Model evaluation and evaluation of sample size

As showin in Figure 3.5, sample sizes of 200 cattle and above were able to recover pre-determined estimates of diagnostic test sensitivities/specificities and prevalence with

reasonable precision for each sampling period. According to this and based on the logistics of carrying out the fieldwork a sample size of 200 for each sampling period was selected.

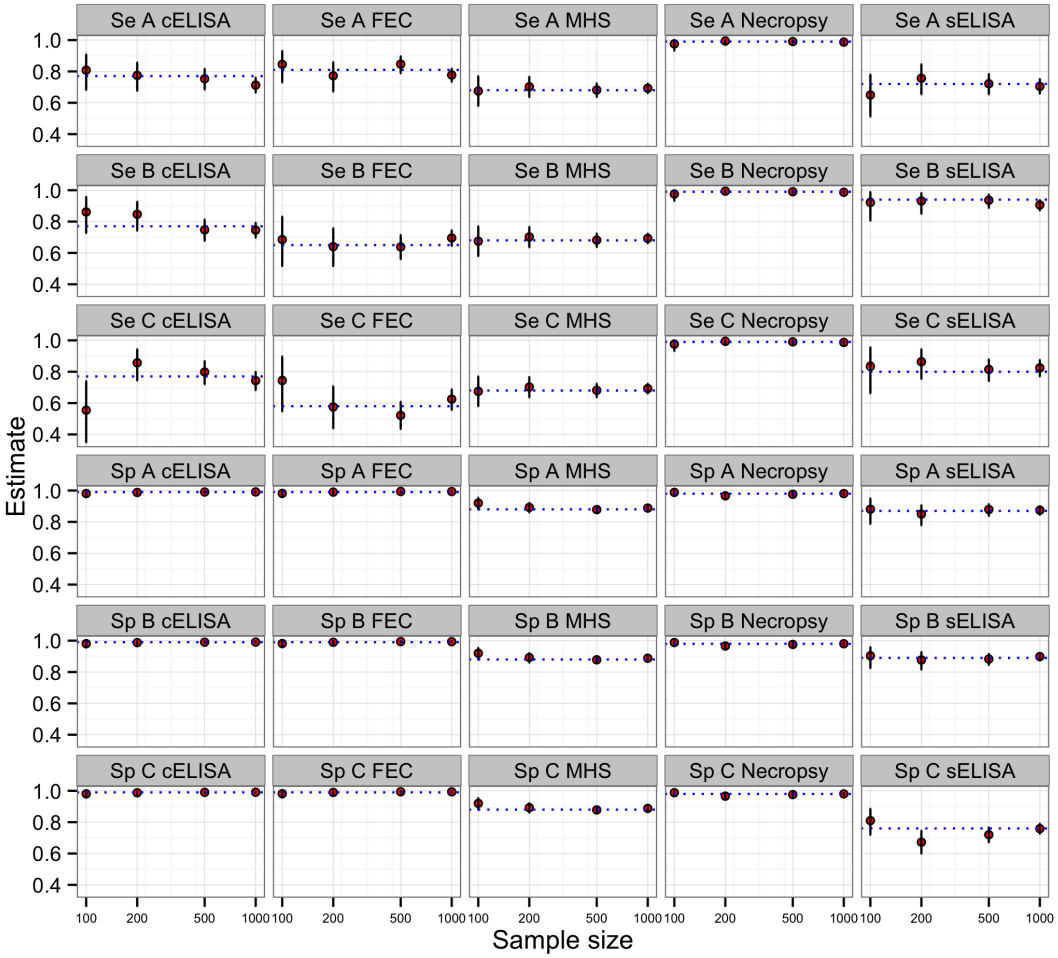


Figure 3.5: Estimation of diagnostic test characteristics against set values (dotted lines) to identify apprpriate sample size. The plot shows for each set parameter (sensitivity (Se) and specificity (Sp)) the estimated value and credibility intervals for each population under a range (100-1000) of simulated samples.

3.4.4 Estimates of diagnostic test sensitivity and specificity

Figure 3.6 is a plot of mean estimates and 95% Bayesian Credible Interval (BCI) for each model parameter. The precise mean estimates and 95% BCIs for each variable are

shown in Table 3.5. *F. hepatica* infection prevalence during summer 2013, winter 2014 and autumn 2014 sampling periods was estimated to be 0.38, 0.31 and 0.23 respectively. Liver necropsy was, as expected, a near perfect test with a sensitivity estimate of 0.99 and a specificity of 0.98. Liver inspection by the abattoir Meat Hygiene Service had a sensitivity estimate of 0.68 and a specificity of 0.88. The sensitivity estimates of the copro-antigen ELISA were allowed to vary between seasons, but were estimated as 0.77 for all three sampling seasons. cELISA was estimated to have a very high specificity of 0.99. The Faecal Egg Count sensitivity values varied greatly between sampling seasons and were estimated as 0.81, 0.77 and 0.58 respectively. The test was shown to be highly specific, 0.99. Lastly, both the sensitivity and the specificity of the serum antibody ELISA were allowed to vary between seasons. Sensitivity estimates varied between seasons with the mean sensitivity estimate being much higher during the winter sampling, 0.94, compared to 0.72 and 0.80 during the summer and autumn sampling periods respectively. Similarly the mean specificity estimate during the autumn sampling of 0.76 was comparatively lower than summer and winter estimates which were 0.87 and 0.89 respectively. The exact data used for this model can be found in Table B.1 in Appendix B.

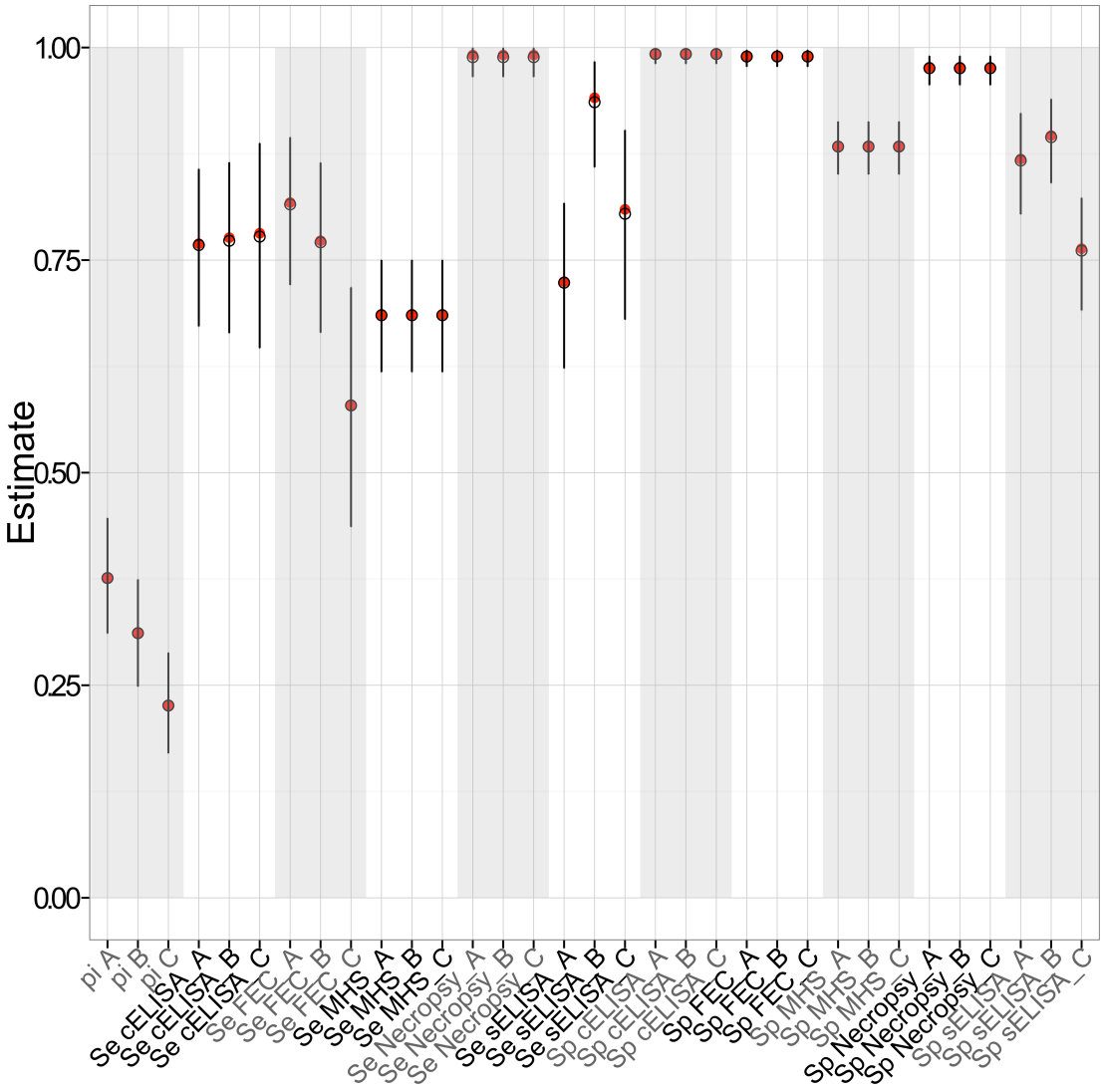


Figure 3.6: Mean posterior estimates and 95% BCIs. Estimates of the prevalence (pi), sensitivity (Se) and specificity (Sp) for each period (summer 2013 (A), winter 2014 (B), autumn 2014 (C)).

Table 3.5: Mean estimates and 95% BCIs of the prevalence and test sensitivity and specificity per period.

Estimate (Season)	Mean	2.5% BCI	97.5% BCI	Estimate (Season)	Mean	2.5% BCI	97.5% BCI
Prevalences							
Summer 2013 (A)	0.38	0.31	0.45				
Winter 2014 (B)	0.31	0.25	0.38				
Autumn 2014 (C)	0.23	0.17	0.29				
Sensitivities				**Specificities**			
MHS inspection	0.68	0.61	0.75	MHS inspection	0.88	0.85	0.91
Necropsy	0.99	0.96	1.00	Necropsy	0.98	0.96	0.99
cELISA (A)	0.77	0.67	0.86	cELISA	0.99	0.98	1.00
cELISA (B)	0.77	0.67	0.87				
cELISA (C)	0.77	0.64	0.88				
FEC (A)	0.81	0.72	0.90	FEC	0.99	0.98	1.00
FEC (B)	0.77	0.66	0.86				
FEC (C)	0.58	0.43	0.72				
sELISA (A)	0.72	0.62	0.82	sELISA (A)	0.87	0.80	0.92
sELISA (B)	0.94	0.86	0.98	sELISA (B)	0.89	0.84	0.94
sELISA (C)	0.80	0.69	0.91	sELISA (C)	0.76	0.69	0.82

3.4.5 Model checking

Appendix B contains figures to demonstrate the results of checking for conditional dependence, the effect of priors and correlation between model variables, respectively. As shown in Figure B.8, there are no major differences in estimates when accounting for covariance for the different combinations of tests and the model with no covariance terms. It was therefore justifiable to use a final model with no covariance terms. Furthermore, Figures B.9 and B.10 show a comparison of prior and posterior distributions which reveals that results are mainly informed by the data. This is further supported by Figure B.11, which presents a comparison between the results presented in the paper and the results of the same model run using non-informative priors for the sensitivities and specificities of all tests, where results do not appear to be altered. Lastly, Figure B.12 presents the cross correlation plots between the parameters included in the model showing that there is no obvious strong correlation between any combination of parameters.

Predictive Values of diagnostic tests

Figure 3.7 shows the positive and negative predictive values of the MHS liver inspection and Feacal egg counts respectively, over a range of prevalences. Estimates for FEC sensitivity was allowed to vary over the 3 sampling seasons hence 3 plots are presented. Prevalence estimates of the 3 sampling periods are shown by dotted lines. It is important to note how predictive values change according to the population prevalence. Additionally, when the PPV values of the two tests are compared at low prevalence levels it is clear that PPV of FEC is higher and varies less than the PPV of MHS due to a much higher specificity estimate for FEC.

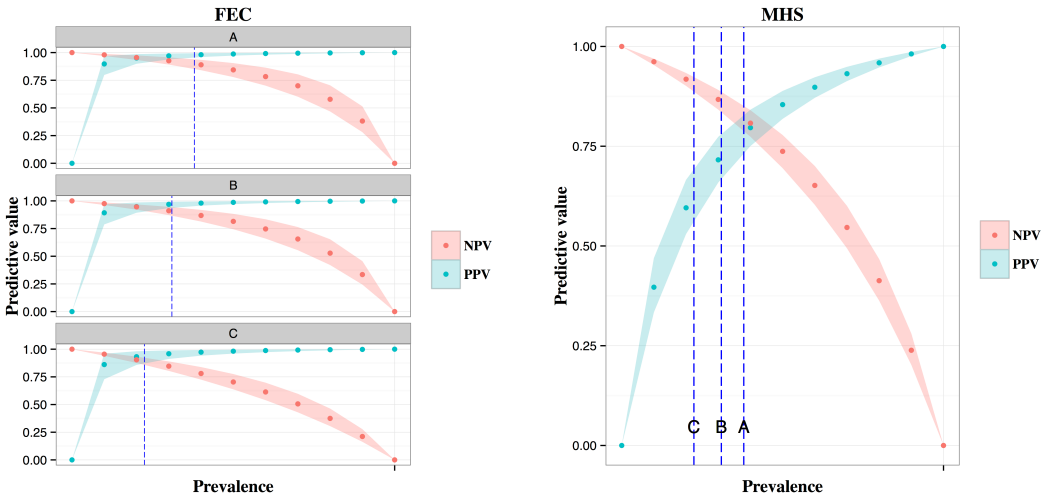


Figure 3.7: Predictive values of FEC (left) and MHS (right) over a range of prevalences. Prevalence estimates for each sampling period are shown by dotted lines.

3.5 Discussion

The main aim of this study was the evaluation of the performance of tests available for the diagnosis of *F. hepatica*. The no gold standard approach introduced by Hui & Walter (146) was used within a Bayesian framework in order to compare the binary results of the five diagnostic tests.

Estimates of sensitivity and specificity for liver necropsy were 0.99 (95% BCI 0.96-1.00) and 0.98 (95% BCI 0.96-0.99) respectively. Liver necropsy is not readily used for disease diagnosis by veterinarians as it is a very time consuming procedure and it can only be carried out post mortem. Its role in this study was to provide a measure of infection and fibrosis levels to better describe the sample. Additionally, as a test previously used as a gold standard in assessments of *F. hepatica* diagnostic tests (76) it was expected to provide near perfect results and therefore be highly informative. A gold standard analysis was not chosen due to the possibility of gall bladder egg sequestration in animals where infections has been successfully treated causing false positive results and very early infections being difficult to detect due to the small size of

flukes causing false negative results. The results of this study show that liver necropsy has a near perfect sensitivity and a very high specificity and must have contributed greatly in the evaluation of the rest of the tests by our model.

Liver inspection is routinely carried out at the abattoir according to Regulation (EC) No 854/2004. The only previously reported estimate of its sensitivity in a European setting identified by the author was from a study in Switzerland by Rapsch et al. (75) which was 63.2%. In the current study the sensitivity estimate of liver inspection appeared to be lower than all other diagnostic tests, except that of FEC during autumn 2014. Similarly, specificity appeared to be similar to the serum antibody ELISA, but lower than all other tests. More precisely the sensitivity was estimated to be 0.68 (95% BCI 0.61-0.75) and the specificity 0.88 (95% BCI 0.85-0.91). Estimates for meat inspection are expected to vary between countries and potentially between abattoirs. It is therefore relevant to report estimates for liver inspection from one of the biggest abattoirs in Scotland as this can provide a way to more accurately estimate the prevalence of *F. hepatica* infection in the UK accounting for imperfectness of this technique. Additionally, liver inspection can provide a useful and practical tool for evaluation of the effectiveness of health planning programmes used on farms. In this setting it is possibly more intuitive to use positive and negative predictive values, which can readily be estimated based on population prevalence as shown in the results section.

Mezo et al. (83) presented a new copro-antigen ELISA which was reported to have a sensitivity of 100% in detecting cattle with fluke burden of two or more parasites and be highly specific with no cross reactivity with parasites including *Moniezia*, *Dicrocoelium*, *Echinococcus* and *Paramphistomum cervi* (83; 85). This ELISA is commercially available by Bio-X Diagnostics in Belgium. The protocol used in the commercial test is a considerable modification of the original, and its performance in the field setting has been poorly assessed, especially in cattle.

In this study sensitivity estimates of the copro-antigen ELISA were allowed to vary between seasons, but were in fact very similar. They were estimated to be 0.77 (95% BCI 0.67-0.86), 0.77 (95% BCI 0.67-0.87), 0.77 (95% BCI 0.64-0.88) during summer 2013, winter 2014 and autumn 2014 sampling periods respectively. These estimates were considerably lower compared to Charlier et al. (76) who reported a sensitivity of 94%. This might be because liver necropsy without detection of eggs in the gall bladder was used as the gold standard, potentially missing a proportion of infected animals and therefore overestimating the sensitivity. Additionally, a lower cut-off than the one recommended in the protocol was used which might increase the sensitivity. Our estimate was similar to that of Palmer et al. (87) who estimated the sensitivity to be 0.80 when the cut-off recommended by the manufacturer was used. When using a lower cut-off Palmer et al. estimated the sensitivity to be 87%.

The specificitiy of copro-antigen ELISA was estimated to be 0.99 (95% BCI 0.98-1.00). This is comparable to Palmer et al. who estimated the specificity to be 1 using the manufacturer's cut off and >99% using their own cut off (87). On the contrary, Charlier et al. estimated the specificity to be 93%. This might be a result of their cut-off adaptation. As the cut off adjustment used by Palmer et al. (manufacturer's cut off multiplied by 0.67) provided greater improvement in the test performance, the model was rerun using the modified cut-off for the cELISA. Sensitivity was estimated as 0.80 (95% BCI 0.71-0.89), 0.85 (95% BCI 0.75-0.93), 0.87 (95% BCI 0.76-0.95) during summer 2013, winter 2014 and autumn 2014 sampling periods respectively. The specificity remained 0.99 (95% BCI 0.98-1.00) confirming that this cut-off modification can improve test sensitivity without compromising specificity. Estimates regarding the other four tests were not altered (results not shown).

Gordon et al. (77) identified rumen fluke from a range of cattle and sheep samples across the UK to be *Calicophoron daubneyi* instead of *P. cervi* which was previously

thought to be the species found in the UK. Even though lack of cross-reactivity with *P. cervi* has already been reported (163), this emphasises that it is important to also check for cross-reactivity of cELISA with *C. daubneyi*. In our study, during the second and third sampling seasons 53 cattle with negative liver necropsy results were found to have at least one fluke in the rumen. None of those samples had a positive copro-antigen result (using both manufacturer's and adjusted cut-off). Rumen flukes collected have not been speciated, but based on the findings of Gordon et al. it is reasonable to assume that a great proportion of those were *C. daubneyi*. This suggests that cELISA does not cross react with this parasite in cattle, which agrees with the results of a similar comparison with cELISA in sheep (77). This is becoming increasingly important in the UK as levels of rumen fluke infection appear to be rising and will further complicate fasciolosis control.

Diagnosis of *F. hepatica* infection by detection of eggs in faecal samples has been around for decades and various protocols exist. The main drawbacks of this, otherwise easy to learn, method are that by definition it can only diagnose patent infections and that it is time consuming and therefore costly or undercharged. It is generally accepted that the specificity of faecal egg counting is almost perfect. In the UK this might be compromised by the increasing prevalence of rumen fluke infection as the eggs are of similar shape (77), even though the trained eye should be able to discriminate between the two kinds of eggs as they are of different colour. As vets and technicians become more aware of the increasing chance of finding rumen fluke eggs in faeces this problem is expected to be reduced. On the other hand, the sensitivity of the test has been reported to vary from well below 50% to moderate values and depends on various factors mainly based on the protocol used, for example volume of faeces (75; 76) and levels of infection in the population (92). In the current context FEC sensitivity was estimated to be 0.81 (95% BCI 0.72-0.90), 0.77 (95% BCI 0.66-0.86) and 0.58 (95% BCI 0.43-0.72) during summer 2013, winter 2014 and autumn 2014 respectively. As expected

the specificity was close to perfect and comparable to the copro-antigen ELISA (0.99, 95%, BCI 0.98-1.00). The sensitivity of FEC was shown to be comparable to cELISA during the first two sampling seasons, while it dropped significantly during autumn 2014. This shows that FEC still remains a very useful test during periods where infection is expected to be mainly chronic, and even superior to antibody ELISA tests as it has a higher specificity. As shown here it is important to remember that when recent infections are expected, for instance at the start of a new liver fluke season, this test performs a lot worse than other tests due to its inability to detect pre-patent infections.

The last test evaluated in this study was the excretory/secretory antibody ELISA developed by the Liverpool School of Tropical Medicine (142). This is the only test included that is developed to also detect past exposure to the parasite. Therefore, both the sensitivity and the specificity were allowed to vary between seasons. Sensitivity appeared to be much higher during the winter sampling, 0.94 (95% BCI 0.86-0.98) when compared to 0.72 (95% BCI 0.62-0.82) and 0.80 (95% BCI 0.69-0.91) during the summer and autumn sampling periods respectively. It was particularly interesting to see whether the false positive rates differed as well. Indeed, specificity during the autumn sampling was estimated to be 0.76 (95% BCI 0.69-0.82), which was comparatively lower than summer and winter estimates of 0.87 (95% BCI 0.80-0.92) and 0.89 (95% BCI 0.84-0.94) respectively. Serum antibody ELISA tests for the diagnosis of *F. hepatica* have been around for decades and have various reported sensitivities and specificities ranging from 91.7% to 100% and 94.6% to 100% respectively (70). The ELISA used in this study is not commercially available and was first presented by Salimi-Bejestani et al. in 2005 with a sensitivity of 98% and a specificity of 96%. For their test evaluation they used FEC positive cattle, while their negative samples came from zero-grazed cattle of no known previous exposure to the parasite. Our sensitivity estimates are much lower and this is thought to be because the test was evaluated

using an abattoir random sample of a range of levels of infection, including ones not detectable by FEC. Similarly, our specificity estimates are lower than previously reported. This is believed to be a result of the inability of antibody ELISAs to distinguish between current and previous exposure as it is highly possible that our sample included animals that were previously infected with the parasite, but which have received treatment, unlike the sample used in the initial evaluation. It is therefore possible that our estimates reflect a more realistic evaluation of this test in the field.

Another issue with serum antibody ELISA tests in general is cross-reactivity with other trematodes (142). The current ELISA showed no cross-reaction with *D. viviparus*, *N. helvetianus* and *O. ostertagi*, while cross-reaction with rumen flukes has not been reported (142). Out of the 53 cattle with rumen flukes identified in the rumen and a negative liver necropsy result, 18 had positive sELISA results. While we cannot know whether those were animals with previous exposure to *F. hepatica* this may be an indication of cross-reactivity which can be further supported by Ibarra et al. (80) who reported cross-reactivity of an ES antigen ELISA first described by Arriaga de Morilla et al. in 1989 with *Paramphistomum* spp. (164).

In our study most animals had a low fluke burden with a mean and median of 8.5 and 4 respectively. Firbrosis scores appeared to be related to burden, but it is important to note that our results explain the limitations of liver inspection by the MHS reflected in its imperfect sensitivity and specificity estimates. Presence of parasites in the liver did not always correspond to obvious fibrosis signs at inspection. Additionally, it is unclear whether “active” or “historic” is a useful classification as many of the livers classified as historic were found to harbour at least one fluke.

Even though knowing whether the infection is absent or present in an animal is highly important, one could argue that the level of infection present could also be important in the control of fasciolosis especially in cattle. As fasciolosis is a chronic disease in

cattle causing mostly sub-clinical disease, it might be meaningful to farmers to know what the intensity of infection is and how that translates to production losses. This information might therefore be used to decide what treatment strategy if any they might decide to use (92). Such an investigation was beyond the scope of this paper, but it is one definitely worth pursuing to investigate the use of available diagnostic tests in quantifying infection or level of production loss attributed to the infection for a more cost effective control of *F. hepatica* infection in cattle.

The present study has several strengths and limitations. We have used systematically chosen samples from naturally infected animals slaughtered at one of Scotland's biggest abattoirs, therefore obtaining a sample more representative of the field situation than if experimentally infected animals were used (165). Whilst we were not able to use simple random sampling due to logistics, we believe that this sampling method enabled us to represent animals arriving at the abattoir during the whole day. Five different tests were used in order to enable us to run a no gold standard analysis, avoiding the limitations of using an imperfect test as a gold standard. This approach certainly does not come without biases. In order to determine whether our proposed model could reclaim tests parameters using the sample size available, test results were simulated for three sub-populations of animals, representing the three sampling periods, under a range of plausible diagnostic test sensitivities and specificities. The model was run using these data and was able to recover pre-determined estimates of diagnostic test sensitivities/specificities and prevalence with reasonable precision for each sampling period. Furthermore, we checked for conditional dependence between tests and carried out appropriate MCMC diagnostics. Moreover, this is the first study to provide information on the appropriateness of available diagnostic tests during three different seasons, even though a first attempt at this was carried out by Charlier et al. (76) using two sampling seasons, a much smaller dataset and a gold standard analysis.

Limitations of this study include the fact that we have not been able to account for differences in meat inspection results depending on which meat inspector carried out the inspection, as well as the fact that seasonal differences were described only during one year. If results of tests are dependent on the liver fluke life cycle, which in turn is heavily dependent on climatic factors, the appropriateness of diagnostic tests in each season might need to be tested over more years to confirm the differences or similarities described here. Additionally, we have assessed the assumption of conditional independence using pairwise dependency models. It is important to bear in mind that it is possible that more complicated dependencies might exist, which we were unable to account for. Nevertheless, due to the fact that the tests compared are looking for five different signals; flukes, fluke damage, eggs, faecal antigens and serum antibodies, it is unlikely that there are biologically likely common proxies of disease that might result in important covariance structures. This is supported by the absence of any considerable change in estimates using the 10 possible covariance pairs.

Overall, our study has provided a valuable insight in the performance of tests available for the diagnosis of *F. hepatica* infections in a population of cattle believed to be representative of the field situation. Knowing its limitations and being able to adjust for them, abattoir liver inspection, can be a valuable tool in monitoring and understanding the changing epidemiology of *F. hepatica* as well as evaluating farm health plans. Faecal egg counting has been shown to still be a valuable tool in the diagnosis of current *F. hepatica* infections, but one has to bear in mind that it is a weak test during periods where recent infections are expected. The copro-antigen ELISA is a comparable test that can be used throughout the year, with evidence to suggest that there is no cross-reaction with the increasingly prevalent rumen fluke parasite. This study also provided further evaluation of an in house ES antigen ELISA showing that while being a valuable test, its sensitivity and specificity estimates are lower in the field setting that previously reported. Liver fluke control is becoming increasing challenging in the UK,

hence the qualitative and quantitative evaluation of available diagnostic tests, as well as development of better ones is an area where ongoing investigation is required.

Chapter 4

Estimation of the delay in reaching slaughter weight in beef cattle infected with F. hepatica using different diagnostic measures

4.1 Abstract

Fasciolosis is a multi-host parasitic disease caused by *Fasciola* spp., affecting livestock and people in many countries worldwide. The typically low levels of infection in cattle mainly manifest as a sub-clinical disease, resulting in indirect production losses, which are difficult to estimate. The lack of obvious clinical signs commonly results in these losses being attributed to other causes such as poor weather conditions or under nutrition. To date it remains unclear at what levels parasite burden leads to production losses, or how the risk of losses can be quantified using diagnostic tests. This study aimed to address these uncertainties by estimating the effect of infection on UK beef

cattle productivity and investigating the use of diagnostic tests in a quantitative manner. We fitted linear regression models for the time taken to reach slaughter weight, based on age, weight and different measures of the liver fluke status, taking sex, breed, season, year and the random effect of farm from which animals were consigned into account. Data for this model were sourced from Scotland's largest red meat abattoir throughtout 2013 and 2014. The model estimates indicated that cattle that had been classified as having liver fluke damage based on routine liver inspection had on average 10 (95% CI 9-12) days greater slaughter age, assuming an average carcass weight of 345 kg. Using a subset of the data, we included the liver fibrosis score (0-3) as a proxy for severity of fluke infection. Estimates from this second model indicated that the increase in age at slaughter was more severe for higher fibrosis scores. The increase in age at slaughter was 34 (95% CI 11-57) days for fibrosis score of 1, 93 (95% CI 61-127) days for fibrosis score 2 and 78 (95% CI 32-125) days for fibrosis score 3. Similarly, comparing different burden categories with animals with no fluke burden, there was a 31 (95 % CI 6-56) days increase in slaughter age for animals with 1 to 10 parasites and 77 (95% CI 31-124) days increase in animals with more than 10 parasites found in their livers. Furthermore, we have used ROC curves to evaluate the ability of a serum antibody ELISA, a copro-antigen ELISA and faecal egg counting to quantify liver fluke infection. Results suggest that the aforementioned tests can be useful in distinguishing between animals with production limiting levels of fibrosis and fluke burden and animals with low or no liver damage or liver fluke burden. Overall, this study provided improved knowledge on the effect of *F. hepatica* infection on beef cattle production in the UK as well as evaluated the ability of available diagnostic tests to be used quantitatively in order to identify heavily infected animals. This information is essential in order to inform targeted treatment strategies and encourage more producers to adopt appropriate control measures.

4.2 Introduction

Fasciolosis, caused by trematode parasites of the genus *Fasciola*, is an infection of worldwide importance, which can affect multiple mammalian species including humans (122; 116). In the UK, fasciolosis is caused by *Fasciola hepatica* (143) and an increase in both its incidence and geographical spread has been described in the last decade. This has been related to climate change and extensive animal movements (166; 167). Fasciolosis is claimed to cost the UK cattle industry £23 million annually (56), a figure that remains a crude estimate as the true effect on production is unclear. At the same time, the incidence of fasciolosis in the UK and in the rest of the central and north-western Europe has been predicted to increase in the coming decades due to climate change (17; 49).

Cattle are less susceptible to showing clinical signs of fasciolosis compared to smaller ruminants, with a higher infection challenge of metacercariae required to cause clinical disease (168; 7). This is thought to be due to the large size of the liver, which leads to a greater functional reserve, and to its more fibrous texture (7). Hence, fasciolosis in cattle mainly manifests as a sublinical chronic disease, associated with hepatic damage and blood loss caused by blood sucking parasites feeding in the bile ducts (8). Additionally, unlike sheep, there is a suggestion that cattle can develop partial immunity with age (5). This is supported by work by Cawdery et al. (169), who showed that the effects of superimposed infection are significantly less deleterious despite the fact that they remain substantial. At the same time, risk of infection appears to increase with age, further supporting the hypothesis that immunity does not prevent re-establishment of new infection (166).

While subclinical infections cause reduced production levels and may contribute to pasture contamination, it is difficult to convince farmers to invest in the control of fas-

ciolosis without demonstrating the clear economic cost of subclinical disease (169). According to Sykes et al. (170), it has been difficult to estimate the effects of subclinical infection due to the lack of data on the size of burden and quantitative production data.

The impact of parasitic infections on production depends on the severity of challenge, the duration of exposure, the effect on metabolism, host immunity, and the metabolic cost of a competent immune system (171). In the case of fasciolosis, reduced cattle growth rates are thought to arise from blood loss during the chronic stages of disease which is replaced using energy that would otherwise be available for production purposes, resulting in reduced feed conversion efficiency (172; 8). Nevertheless, Hicks et al. (173) argue that reduction in weight gain results from reduced feed intake and not reduced feed efficiency.

Studies on the impact of liver fluke infection on production parameters have presented conflicting results (168). A few early studies failed to show a negative effect of infection in cattle such as those by Knapp et al. (174) and Owen (175) published in 1971 and 1984 respectively, while a number of studies carried out later studies showed a negative effect of fasciolosis. Hick et al. (173), in a study performed in Oklahoma, reported that feedlot steers infected with liver fluke gained weight 5.9% slower than non infected steers during a 135 day feeding period and had, on average, 8.6 kg lower mean carcass weights. In a trial of 69 beef calves, (169), showed that as few as 54 flukes per animal could cause an 8% reduction in weight gain during the first 6 months post infection, with greater effects on production observed with higher burdens. Genicot et al. (176), in a study in Belgium, reported an 18% increase in weight gain in flukicide-treated double-muscled Belgian Blue cattle compared to untreated controls. Similarly, Marley et al. (177), in a study of 120 crossbred steers, did not identify a significant difference in weight gain during the immature phase of infection when the

animals were on grass, but reported improved weight gain in the treated vs. untreated groups of infected steers during the mature stages of infection when the animals were housed. A more recent study in Louisiana, by Loyacano et al. (178), reported a 6% body weight increase in crossbred beef heifers in a group treated for liver fluke compared to an untreated group (92).

Two recent abattoir studies carried out by Charlier et al. (179) in Belgium and Sanchez-Vazquez and Lewis (60) in Scotland have investigated the effect of liver fluke infection on carcass characteristics. The first study included 1450 Belgian Blue suckler cows and used the results of a meat juice antibody ELISA to estimate the liver fluke status of each animal. While they failed to identify a statistically significant association between *F. hepatica*-specific antibody levels and carcass characteristics including weight, conformation and fatness scores at the individual animal level, they observed that an increase over the interquartile range of the mean herd ELISA results was associated with a 3.4 kg lower mean herd carcass weight. The study by Sanchez-Vazquez and Lewis (60) is probably the most comprehensive study to date, including data from 328,137 cattle of various beef breeds over a six year period. Adjusting for other important factors such as for an animal's age, breed and sex, season and year, they showed that liver fluke infected cattle had 0.63 kg lower cold carcass weight and poorer carcass conformation scores.

Most of the studies on the effect of fasciolosis on beef production have involved experimentally infected animals or limited types of breeds and are hence unlikely to correspond to naturally infected cattle populations. Our study will analyse data on prime cattle slaughtered at Scotland's largest red meat abattoir collected over a two year period with the aim of:

a) Estimating the difference in slaughter age between beef cattle infected with liver fluke vs. uninfected cattle using meat inspection results as an indication of in-

fection status.

b) Estimating the difference in slaughter age of beef cattle with different liver fibrosis scores used as an indication of the extent of liver fluke infection.

c) Estimating the difference in slaughter age of beef cattle with different levels of parasite burden as an indication of the extent of liver fluke infection.

d) Investigating the quantitative use of diagnostic tests in distinguishing between animals of high and low levels of liver fluke infection.

4.3 Materials and methods

4.3.1 Data sources

Data routinely collected at the abattoir

This refers to a two year (2013-2014) abattoir dataset, which included data routinely collected at Scotbeef abattoir such as results of inspection by the Meat Hygiene Service (MHS), i.e. reasons for offal or carcass rejection, along with carcass characteristics such as carcass weight and grade. For the purposes of this study results of liver inspection by the MHS were used as an indication of liver fluke infection. Further information on the routine collection of data at Scotbeef abattoir can be found in chapter 2.

Abattoir based sampling

The second dataset used in this study is a combination of data collected using abattoir based sampling and the two year dataset described above. A detailed description of the sampling strategy can be found in chapter 2. Briefly, samples, including blood, faeces, whole livers and gall bladders from each animal, were collected during three sampling periods; June - July 2013, January - March 2014 and August - October 2014 in order to be tested with five liver fluke diagnostic tests. The diagnostic tests used in this chapter are mentioned below.

4.3.2 Diagnostic tests

Liver necropsy

For the purposes of this study, liver necropsy was used in order to assign each liver a fibrosis score as well as to count the number of flukes present. As it was important to assign a fibrosis score in a manner that could replicate that of the meat inspectors on the line, fresh incisions were made parallel to and approximately 1 cm away from the meat inspector's incisions. Fibrosis scores 0 to 3 (no, mild, moderate and severe) were assigned with score 0 referring to a liver with no signs of fibrosis, 1 to a liver with mild focal fibrosis, 2 to a liver with severe local or mild generalised fibrosis and 3 for a liver with severe local fibrosis and calcified bile ducts or severe generalised fibrosis. In order to count the number of parasites present the liver was sliced into 1-2 cm slices, soaked in hot water and flukes where collected throughout by squeezing each slice. The procedure was adapted from Clery et al. (27) and De Bont et al. (141) and the full description is included in chapter 2.

Faecal egg count (FEC)

A quantitative sedimentation technique was performed as described in chapter 2 to estimate the number of *F. hepatica* eggs per gram in the faecal samples collected. These were clearly differentiated from rumen fluke eggs based on the fact that *F. hepatica* eggs are more regularly shaped and show bile staining.

Copro antigen ELISA (cELISA) and serum antibody ELISA (sELISA)

A commercially available *F. hepatica* antigen ELISA kit (Bio-X Diagnostics, Belgium) was used to test faecal samples for the presence of excretory-secretory anti-

gens, following the manufacturer's instructions (83). Furthermore, an in-house excretory/secretory (ES) antibody ELISA, developed by the Liverpool School of Tropical Medicine (142), was used to test serum samples for the presence of antibodies against *F. hepatica*. For the purposes of this study, the percentage positive results of each test were used. The detailed protocol followed for each test as well as the equation used to estimate the percentage positive result is described in chapter 2.

4.3.3 Statistical analysis

Mixed effects regression model

We used linear mixed effects regression models to estimate the difference in cattle age at slaughter according to fluke status at an average slaughter weight, while controlling for differences due to sex, breed, season, year and farm-level variation. In order to make the interpretation of the models more straightforward and to account for the fact that an animal of 0 weight would not be meaningful we centered the predictor variable weight around its mean (180). Additionally, as more than one animal included in the model came from each producer, cattle coming from the same farm could not be assumed to be independent, hence the farm each animal was consigned from was introduced in the model as a random effect. The models had the following general format, where α is the fixed intercept, β is the fixed effect of each covariate and μ is the random effect of each farm (*i*).

$$\text{Age at slaughter} \sim \alpha + \beta_1 * \text{weight.centered} + \beta_2 * \text{fluke} + \beta_3 * \text{breed} + \beta_4 * \text{sex} + \beta_5 * \text{season} + \beta_6 * \text{year} + \beta_7 \text{fluke} * \text{weight.centered} + \beta_8 * \text{breed} * \text{weight.centered} + \beta_9 * \text{sex} * \text{weight.centered} + \beta_{10} * \text{season} * \text{weight.centered} + \beta_{11} * \text{year} * \text{weight.centered} + \mu_i * \text{farm}$$

Three regression models were fitted using three different measures of liver fluke infec-

tion. In the first model, the "MHS model", we used results of liver inspection by the MHS as a binary indicator of disease (two levels; liver rejected due to signs of liver fluke, liver not rejected due to signs of liver fluke). This model used the entire 2 year abattoir dataset. In the second model, the "fibrosis model", we used fibrosis scoring recorded during liver necropsy, carried out by the author, as a categorical indicator of severity of disease (four levels; fibrosis scores 0 to 3). For the final model, the "burden model", we used 3 different burden categories (0, 1-10 and more than 10 *F. hepatica* parasites) found in the liver during liver necropsy as a measure of liver fluke infection. The last two models used the second dataset, which included results of the abattoir based sampling and a subset of the routinely collected abattoir data.

The R statistical software (155) was used for this analysis using scripted procedures to ensure robust and replicable analysis. R package *lme4* (181) was used for the regression analysis and confidence intervals for parameter estimates were computed using the bootstrapping method provided by the same package. Models were visually assessed for normality of the random effect, normality and homoscedasticity of the residuals as well as model fit using package *predictmeans* (182).

Spearman correlation coefficient

We investigated the value of FECs and the two ELISA-based tests in quantifying *F. hepatica* infection measured by fluke burden and fibrosis score recorded at liver necropsy. We calculated the Spearman rank correlation coefficient between the number of recovered parasites in the liver and the serum and copro-antigen ELISA PP values as well as the number of eggs per gram counted in the faecal samples. Similar analysis was carried out comparing the fibrosis score reported for each liver during necropsy (183; 85; 76) with the FECs and the ELISA-based tests. R package *pspearman* (184) was used for this analysis and *ggplot2* (158) was used to plot the aforementioned rela-

tionships.

Receiver operating characteristic (ROC) curves

ROC curves are a tool commonly used to evaluate the ability of diagnostic tests and statistical models to distinguish between animals of two different states, for example infected vs. not infected. A ROC curve is a plot of the probability of a test classifying an infected animal as infected (sensitivity) vs. the propability of classifying an uninfected animal as infected (1-specificity) at all possible test cut-off values, in other words the true positive rate vs. the false positive rate at different cut-offs. The closer this curve is to the top left corner, the better the diagnostic test performance. The closer the curve to the y=x diagonal, the closer the results of this test would be to the ones obtained by chance. ROC analysis can be used to calculate the overall predictive ability of the test, which is referred to as the area under the curve (AUC). AUC essentially provides a summary of all the possible combinations of sensitivity and specificity dependend on test cut-offs. ROC analysis can also be used to identify the test cut-off where the test would have optimal combination of sensitivity and specificy (185; 186; 187; 188). For the purposes of this work ROC curves were used in order to evaluate the ability of the three diagnostic tests to distinguish between animals with; a) high vs. low or zero levels of fibrosis and b) high vs. low or no fluke burden. In this study, high burden refers to a fluke burden of more than 10 parasites, a cut-off suggested by Charlier et al. as the new economic threshold (76). The R package *Epi* (189) was used to compute values for the ROC curves, which were plotted using ggplot2 (158).

4.4 Results

4.4.1 Descriptive results

Routine abattoir data

A large dataset containing data on 169,605 cattle was made available by Scotbeef and used for the MHS model. The dataset contained animals that were slaughtered at Scotbeef abattoir between 2013-01-03 and 2014-11-10, with the age of the animals ranging from 366 to 1199 days at the time of slaughter. Information on sex was available for 162,744 of these cattle; 59,321 were female and 103,423 were male. Cattle were sourced from 1,724 different producers. Overall, 45,452 cattle had livers rejected by the MHS service due to signs of liver fluke. Figures 4.1 and 4.2 show the age distribution of these animals according to liver fluke status based on the results of the liver inspection by the MHS alone and by breed. Table 4.1 shows the numbers and percentages of livers rejected due to signs of liver fluke by different cattle breed and sex. As can be seen from Figures 4.1 and 4.2, the mean age at slaughter was found to be greater for animals with livers rejected due to liver fluke when compared to animals that did not have their livers rejected, irrespective of the breed of the animal.

Results from the abattoir based sampling

619 cattle were sampled during the three sampling periods sourced from 255 different producers. Sampled cattle had a slaughter age range of 369 to 1,121 days. Sex was available for 589 of the animals sampled of which 215 were female and 374 were male. Table 4.2 shows the various breeds and sex of sampled animals by fibrosis score. One or more parasites were identified in 164 of the 619 animals sampled and fluke burden ranged from 1 to 86 with a mean of 9 and a median of 4 (Figure 4.3). Figures 4.4

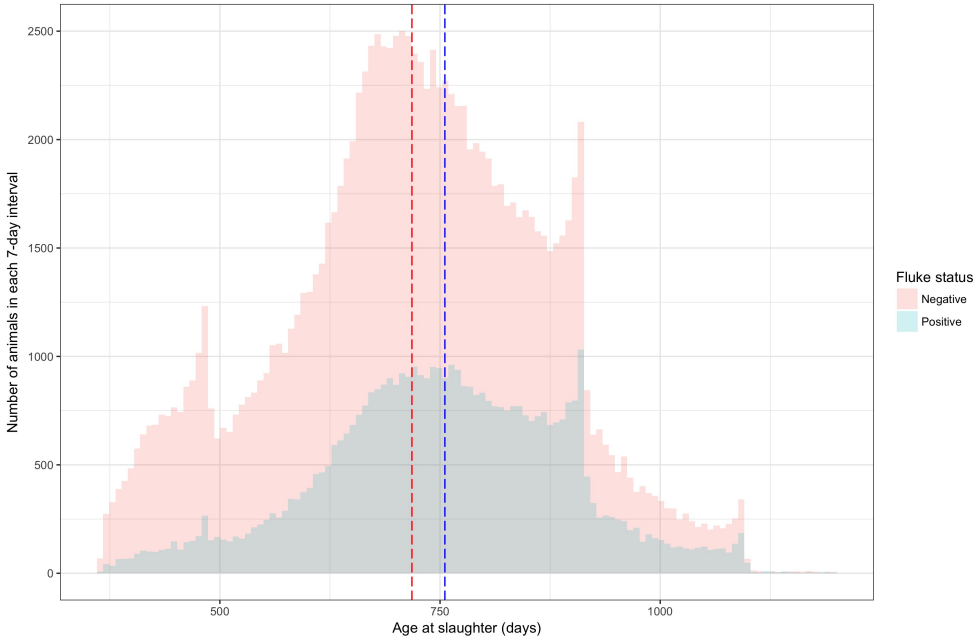


Figure 4.1: Distribution of age of cattle at slaughter by liver fluke status. This figure compares the age distribution at the time of slaughter between animals which had livers rejected due to signs of liver fluke infection and those which did not using data from the two year abattoir dataset (n=169,605). Dotted lines show the mean ages at which fluke negative (red) and fluke positive (blue) cattle are slaughtered. There is a 37 days difference between the two means.

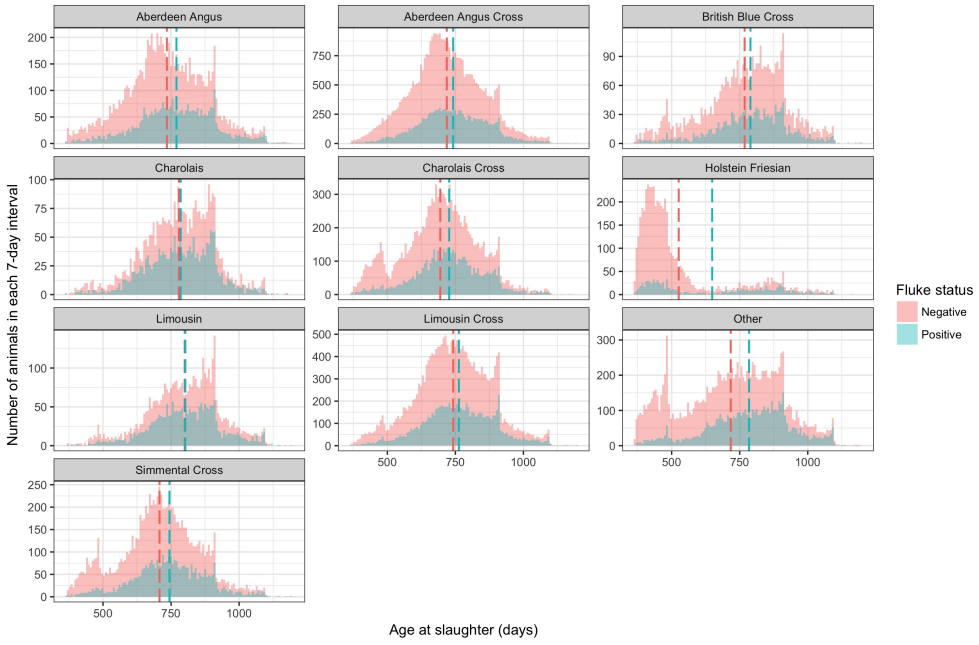


Figure 4.2: Distribution of age of cattle at slaughter by breed and liver fluke status. This figure compares the age distribution at the time of slaughter for different breeds between animals which had livers rejected due to signs of liver fluke infection and those which did not using data from the two year abattoir dataset (n=169,605). Dotted lines show the mean ages at which fluke negative (red) and fluke positive (blue) cattle are slaughtered. The difference in age of positive and negative animals varies between different breeds, but the mean age of fluke positive animals remains greater than or equal to fluke negative animals of all breeds.

and 4.5 show the slaughter age distribution of these animals according to their fibrosis score and parasite burden levels respectively. Figure 4.6 shows the proportion of liver fibrosis scores observed in each age group.

Table 4.1: Distribution of animals with livers rejected due to liver fluke, across the different breeds and across the sex of the animals.

Variable	Levels	No of livers rejected	Percentage rejected	Total
BREED	Aberdeen Angus	3,477	27.6%	12,602
	Aberdeen Angus Cross	12,901	24.8%	52,032
	British Blue Cross	1,452	26.0%	5,577
	Charolais	1,906	35.7%	5,332
	Charolais Cross	5,123	28.1%	18,201
	Holstein Friesian	1,018	16.4%	6,197
	Limousin	2,152	35.2%	6,105
	Limousin Cross	8,408	27.7%	30,359
	Other	5,419	27.4%	19,781
	Simmental Cross	3,596	26.8%	13,419
SEX	Female	16,458	27.7%	59,321
	Male	26,814	25.9%	103,423

Table 4.2: Distribution of levels of observed fibrosis scores, depending on breed and depending on the sex of the animal.

Variable	Levels	0	1	2	3	Total
BREED	Aberdeen Angus	25	11	6	2	44
	Aberdeen Angus Cross	120	35	8	11	174
	British Blue Cross	13	1	3	1	18
	Charolais	4	7	1	0	12
	Charolais Cross	41	19	9	3	72
	Holstein Friesian	28	3	1	1	33
	Limousin	10	11	4	1	26
	Limousin Cross	68	32	12	6	118
	Other	46	22	14	3	85
	Simmental Cross	26	6	3	2	37
SEX	Female	125	56	23	11	215
	Male	241	82	32	19	374

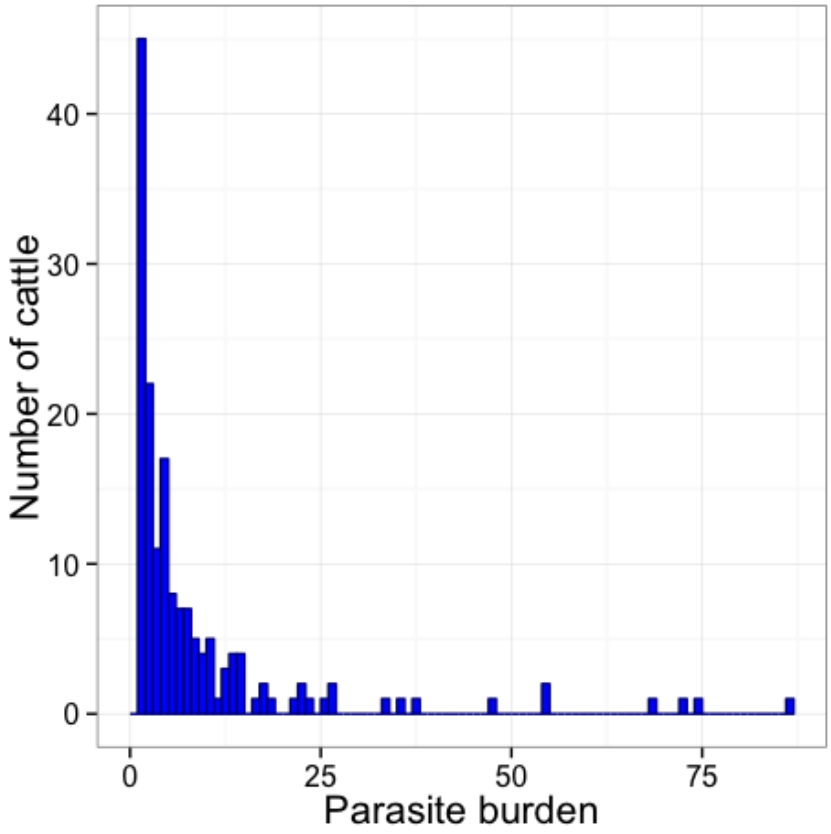


Figure 4.3: Distribution of parasite burden among tested cattle. Figure included animals with a parasite burden greater than 0 (n=164). The distribution is positively skewed with very few animals with burdens of more than 25 parasites.

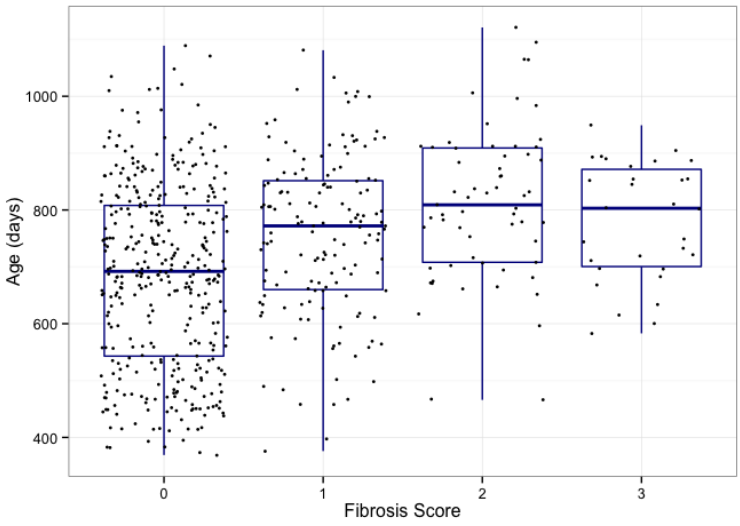


Figure 4.4: Distribution of age of cattle at slaughter by fibrosis score. Figure shows box plots and actual values of cattle age at slaughter for each fibrosis score (n=619). There is a positive age trend as fibrosis score increases from 0 to 3, but there is great variability of age values for each score.

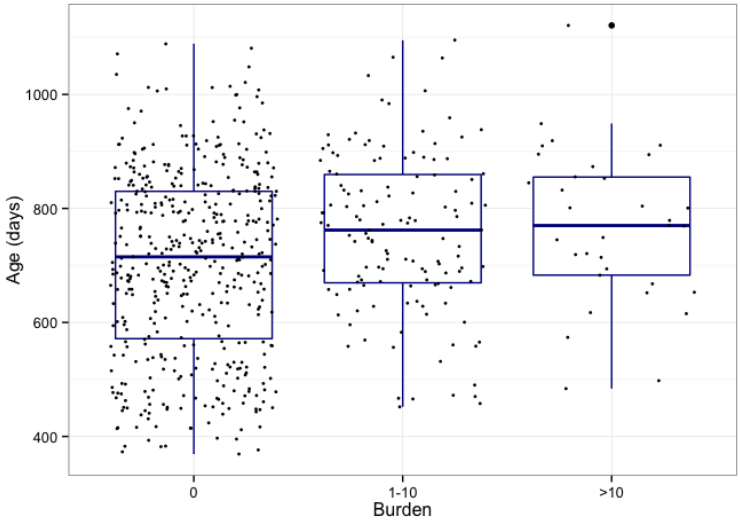


Figure 4.5: Distribution of age of cattle at slaughter by burden category. Figure shows box plots and actual values of cattle age at slaughter for each burden category (n=619). Median slaughter age is higher in animals with a burden of more than 0, but the slaughter age distributions of animals of burden 1-10 and >10 are very similar.

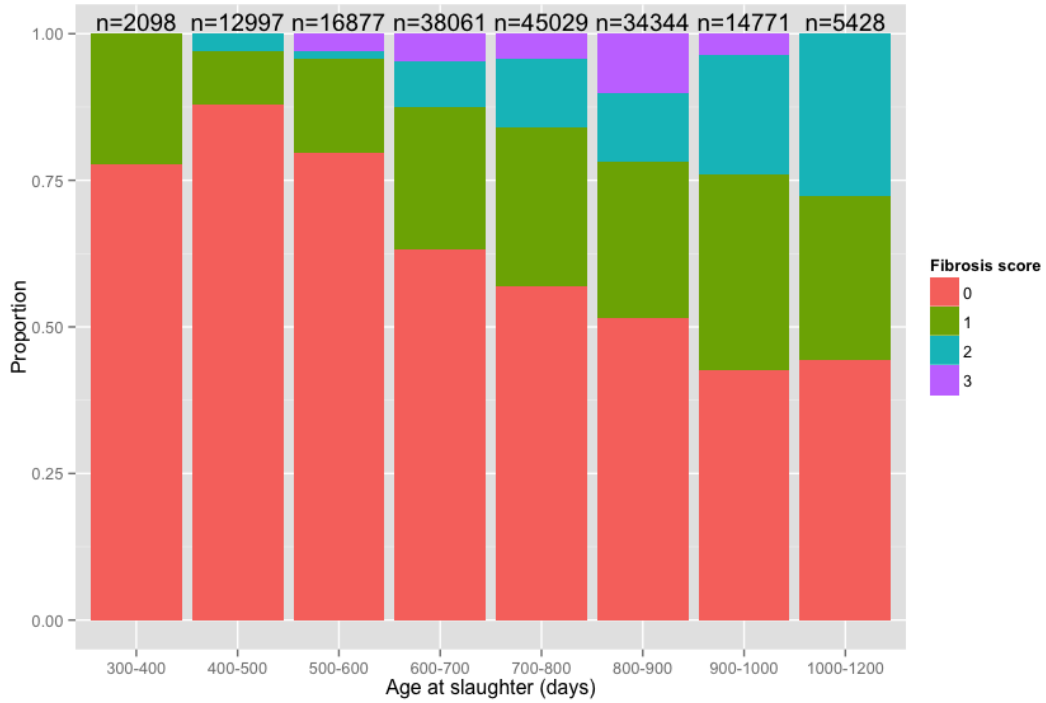


Figure 4.6: Proportion of animals with different fibrosis score, by age. Figure shows the proportion of liver fibrosis scores observed in each age group (n=619). The proportions of fibrosis scores 1 and above increase as slaughter age increases.

4.4.2 Regression modelling results

The MHS model used the full 2013-2014 abattoir dataset. Using the results from liver inspection by the MHS as an indicator of infection, the model indicated that a present liver fluke infection had a noticeable effect on the time to reach slaughter weight. More precisely an animal with an average carcass weight of 345 kg took on average 10 (95% confidence interval 9-12) days longer to reach slaughter weight if it had its liver rejected due to liver fluke compared to animals who did not have their livers rejected due to fluke. The estimates of the MHS model parameters can be seen in Table 4.3.

Both the fibrosis and the burden models used the second dataset which included data from the abattoir based sampling. The fibrosis model, using fibrosis score as an indicator of severity of disease, showed that animals of mean slaughter weight of 345 kg took 34 (95% CI 11-57) days longer to finish if they had a fibrosis score of 1, 93 (95% CI 61-127) days longer with a fibrosis score 2 and 78 (95% CI 32-125) days longer with a fibrosis score of 3, when compared to cattle with fibrosis score 0 (Figure 4.7). Similarly, the burden model showed an increase of 31 (95% CI 6-56) days for animals with a parasite burden of 1-10, and 77 (95% CI 31-124) days for animals with a parasite burden of more than 10 when compared with animals with no parasites found in their livers (Figure 4.8). Point estimates and confidence intervals for both models are shown in Tables 4.4 and 4.5 respectively.

Model diagnostic plots can be found in Appendix C. The two latter models appear to perform well having normally distributed random effects and residuals, minimal residual heteroscedasticity and a good predictive ability. The MHS model on the other hand performs more poorly and this will be further discussed later.

Table 4.3: MHS model results. Point estimates and confidence intervals for model parameters for estimating average difference of the age at slaughter between animals with and without fluke at the mean slaughter carcass weight of 345 kg.

Variable	Estimate	Lower 95% CI	Upper 95% CI
Intercept	741.20	736.10	746.30
fluke negative	1		
fluke positive	9.89	9.14	11.85
weight.center	0.72	0.69	0.77
sex_femail	1		
sex_male	-38.91	-40.47	-37.90
breed			
Aberdeen Angus	1		
Aberdeen Angus Cross	-4.16	-6.52	-2.09
British Blue Cross	21.10	17.80	25.09
Charolais	-19.10	-26.42	-14.60
Charolais Cross	-35.14	-39.01	-33.12
Holstein Friesian	20.94	15.69	26.58
Limousin	17.30	12.33	21.29
Limousin Cross	11.07	7.92	13.81
Other	21.20	17.97	24.05
Simmental Cross	-2.87	-6.56	0.55
season_Q1	1		
season_Q2	5.09	3.06	6.10
season_Q3	1.99	0.36	3.42
season_Q4	7.08	5.89	8.72
year_2013	1		
year_2014	8.37	7.79	9.64
fluke*weight.center	-0.02	-0.03	-0.0001
sex_male*weight.center	-0.25	-0.26	-0.21
Aberdeen Angus Cross*weight.center	0.08	0.05	0.11
British Blue Cross*weight.center	0.05	0.01	0.11
Charolais*weight.center	0.15	0.08	0.28
Charolais Cross*weight.center	0.14	0.06	0.18
Holstein Friesian*weight.center	0.79	0.71	0.83
Limousin*weight.center	0.05	-0.06	0.11
Limousin Cross*weight.center	0.07	0.01	0.09
Other*weight.center	0.05	0.01	0.06
Simmental Cross*weight.center	0.02	-0.02	0.09

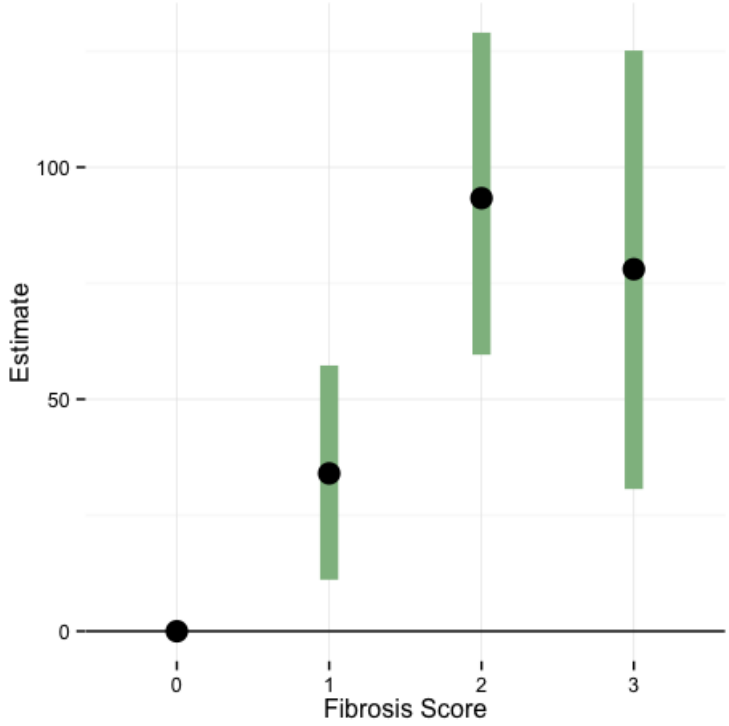


Figure 4.7: Point estimates and 95% Confidence Intervals for different fibrosis scores. Figure shows the estimated increase in slaughter age in days for an animal with 345 kg carcass weight for fibrosis scores 1-3 compared to a fibrosis score of 0, as estimated by the fibrosis model.

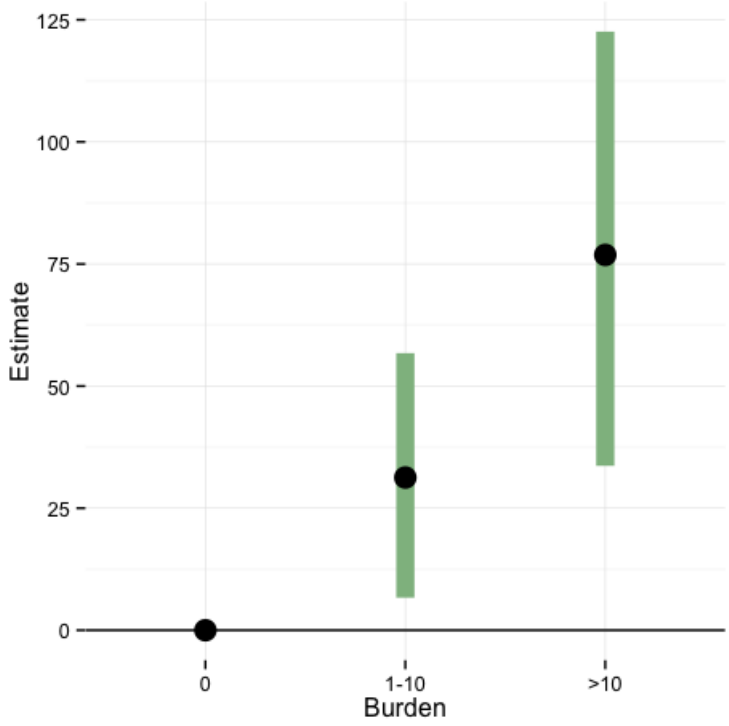


Figure 4.8: Point estimates and 95% Confidence Intervals for animals with different levels of burden compared to animals with 0 burden. Figure shows the estimated increase in slaughter age in days for an animal with 345 kg carcass weight for burden categories 1-10 and >10 compared to a burden of 0, as estimated by the burden model. Confidence Intervals for mean slaughter age at 345 kg carcass weight for animals with different levels of burden, relative to healthy animals.

Table 4.4: Fibrosis model results. Point estimates and confidence intervals for model parameters for estimating average difference of the age at slaughter in animals with different fibrosis scores at the mean slaughter carcass weight of 345 kg.

Variable	Estimate	Lower 95% CI	Upper 95% CI
Intercept	697.20	633.70	761.60
fibrosis score 0	1	-	-
fibrosis score 1	34.02	10.58	57.42
fibrosis score 2	93.33	60.85	127.20
fibrosis score 3	78.04	32.28	124.90
weight.center	0.44	-0.41	1.33
sex_female	1		
sex_male	-41.14	-71.92	-11.32
breed			
Aberdeen Angus	1		
Aberdeen Angus Cross	-25.57	-73.45	20.91
British Blue Cross	-15.96	-85.52	45.68
Charolais	-5.45	-116.50	103.90
Charolais Cross	-43.86	-100.50	9.80
Holstein Friesian	-104.60	-193.60	-20.74
Limousin	44.89	-32.27	125.80
Limousin Cross	-27.97	-81.27	20.20
Other	-24.66	-81.63	28.09
Simmental Cross	-53.81	-112.20	2.48
season_Q1	1		
season_Q2	32.23	-21.60	85.13
season_Q3	24.54	-6.51	56.60
season_Q4	5.60	-38.50	48.98
year_2013	1		
year_2014	63.15	29.51	96.52
fibrosis score)1*weight.center	-0.04	-0.50	0.39
fibrosis score)2*weight.center	-0.23	-0.88	0.43
fibrosis score)3*weight.center	0.27	-1.05	1.54
sex_male*weight.center	-0.21	-0.77	0.31
Aberdeen Angus Cross*weight.center	0.14	-0.74	1.04
British Blue Cross*weight.center	-0.52	-2.01	1.06
Charolais*weight.center	0.36	-3.19	4.19
Charolais Cross*weight.center	0.27	-0.67	1.24
Holstein Friesian*weight.center	0.91	-0.49	2.36
Limousin*weight.center	-0.11	-1.49	1.27
Limousin Cross*weight.center	0.62	-0.24	1.55
Other*weight.center	0.34	-0.62	1.30
Simmental Cross*weight.center	0.41	-0.72	1.58

Table 4.5: Burden model results. Point estimates and confidence intervals for model parameters for estimating average difference of the age at slaughter in animals with different levels of burden at the mean slaughter carcass weight of 345 kg.

Variable	Estimate	Lower 95% CI	Upper 95% CI
Intercept	709.30	643.50	777.60
burden 0	1		
burden 1 to 10	31.27	5.77	56.05
burden >10	76.86	31.05	123.90
weight.center	0.38	-0.44	1.23
sex_female	1		
sex_male	-42.26	-71.95	-13.06
breed			
Aberdeen Angus	1		
Aberdeen Angus Cross	-36.37	-84.22	10.19
British Blue Cross	-27.21	-92.88	41.32
Charolais	4.82	-109.50	118.10
Charolais Cross	-49.96	-102.50	6.20
Holstein Friesian	-124.00	-211.30	-33.73
Limousin	42.09	-40.35	124.50
Limousin Cross	-38.07	-89.33	13.98
Other	-29.73	-83.80	25.46
Simmental Cross	-70.14	-126.60	-7.84
season_Q1	1		
season_Q2	41.77	-10.55	93.30
season_Q3	31.36	-0.97	61.96
season_Q4	9.64	-33.91	55.04
year_2013	1		
year_2014	68.56	34.36	103.30
burden 1 to 10*weight.center	-0.32	-0.77	0.11
burden >10*weight.center	0.93	-0.01	1.89
sex_male*weight.center	-0.20	-0.79	0.33
Aberdeen Angus Cross*weight.center	0.22	-0.65	1.06
British Blue Cross*weight.center	-0.33	-1.87	1.28
Charolais*weight.center	0.21	-3.53	3.97
CharolaisCross*weight.center	0.36	-0.61	1.29
HolsteinFriesian*weight.center	0.76	-0.67	2.18
Limousin*weight.center	0.02	-1.31	1.39
LimousinCross*weight.center	0.69	-0.22	1.58
Other*weight.center	0.35	-0.62	1.30
Simmental Cross*weight.center	0.34	-0.88	1.50

4.4.3 Relationship between diagnostic test results and fluke infection

The number of animals tested and the proportion of animals classified as positive for each of the three *F. hepatica* laboratory diagnostic tests are shown in Table 4.6. Continuous results of the three tests against parasite burden and fibrosis scores are shown in figure 4.9. There is a positive trend between quantitative results of the three tests and a) parasite burden and b) fibrosis score. This is further supported by the Spearman rank correlation coefficients shown in Table 4.7. Nevertheless, test result values at each level of both burden and fibrosis are very variable (figure 4.9).

Table 4.6: Number of animals tested and the measured prevalence for each of the four types of diagnostic tests used.

Diagnostic test	Total tested	Number positive	Prevalence
Liver Necropsy	619	196	0.32
FEC	619	143	0.23
cELISA	619	148	0.24
sELISA	619	223	0.36

Table 4.7: Spearman rank correlation coefficients between the number of parasites in the liver, fibrosis score and continuous test results of each diagnostic test.

Diagnostic Test	Liver fluke burden	Fibrosis score
FEC eggps per gram	0.79	0.57
cELISA PP	0.72	0.54
sEISA PP	0.62	0.67

Figure 4.10 shows the ROC curves evaluating the ability of the 3 diagnostic tests in differentiating between:

1. Animals with fibrosis score 2 or higher vs. animals with fibrosis score 1 or 0.

   At the suggested cut-offs reported on the plot, the serum antibody ELISA and the copro-antigen ELISA have high sensitivities for detecting animals with fibrosis scores 2 or higher (91.9% and 82.6% respectively). Similarly, they have moderate specificities of 79.6% and 79.0% respectively. Faecal egg counts have a relatively low sensitivity of 72.1% at detecting animals with fibrosis scores of 2 or more, while the specificity is slightly higher than that of the other tests (84.8%).

2. Animals with fibrosis score 3 vs. with fibrosis score 2 or less.

   At the suggested cut-offs, both the serum antibody and the copro-antigen ELISA tests have higher sensitivities (93.3% and 90.0% respectively), while the specificities are decreased to 74.6% and 67.5% respectively. As before Faecal egg counts have a low sensitivity of 73.3%, but higher specificity than the other tests (79.3%).

3. Animals with parasite burden of more than 10 vs. animals with parasite burden of 10 or less.

   In terms of current parasite burden all three tests have high sensitivity at detecting animals with more than ten liver flukes (sELISA-96.9%, cELISA-100.0%, FEC-93.8%). Faecal egg counts have a high specificity of 88.7%, while the ELISA based tests have lower specificities (sELISA-75.1%, cELISA-80.4%) at the suggested cut-offs.

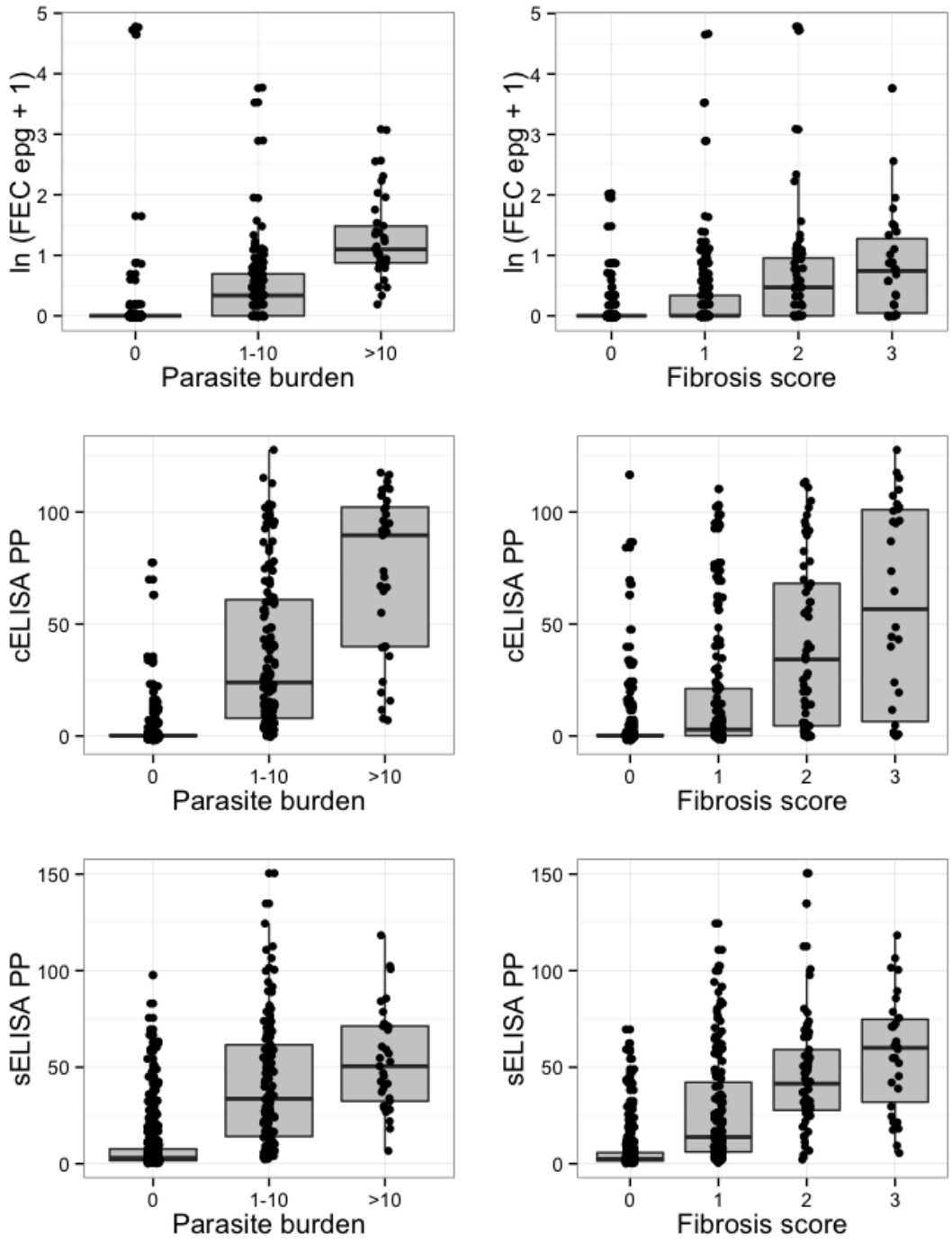


Figure 4.9: Relationship between diagnostic tests and severity of disease. Plots show the raw results of each diagnostic test vs. parasite burden (left) and fibrosis score (right). There are positive trends between test results and increasing parasite burden and fibrosis scores, but there is great variability in actual values.

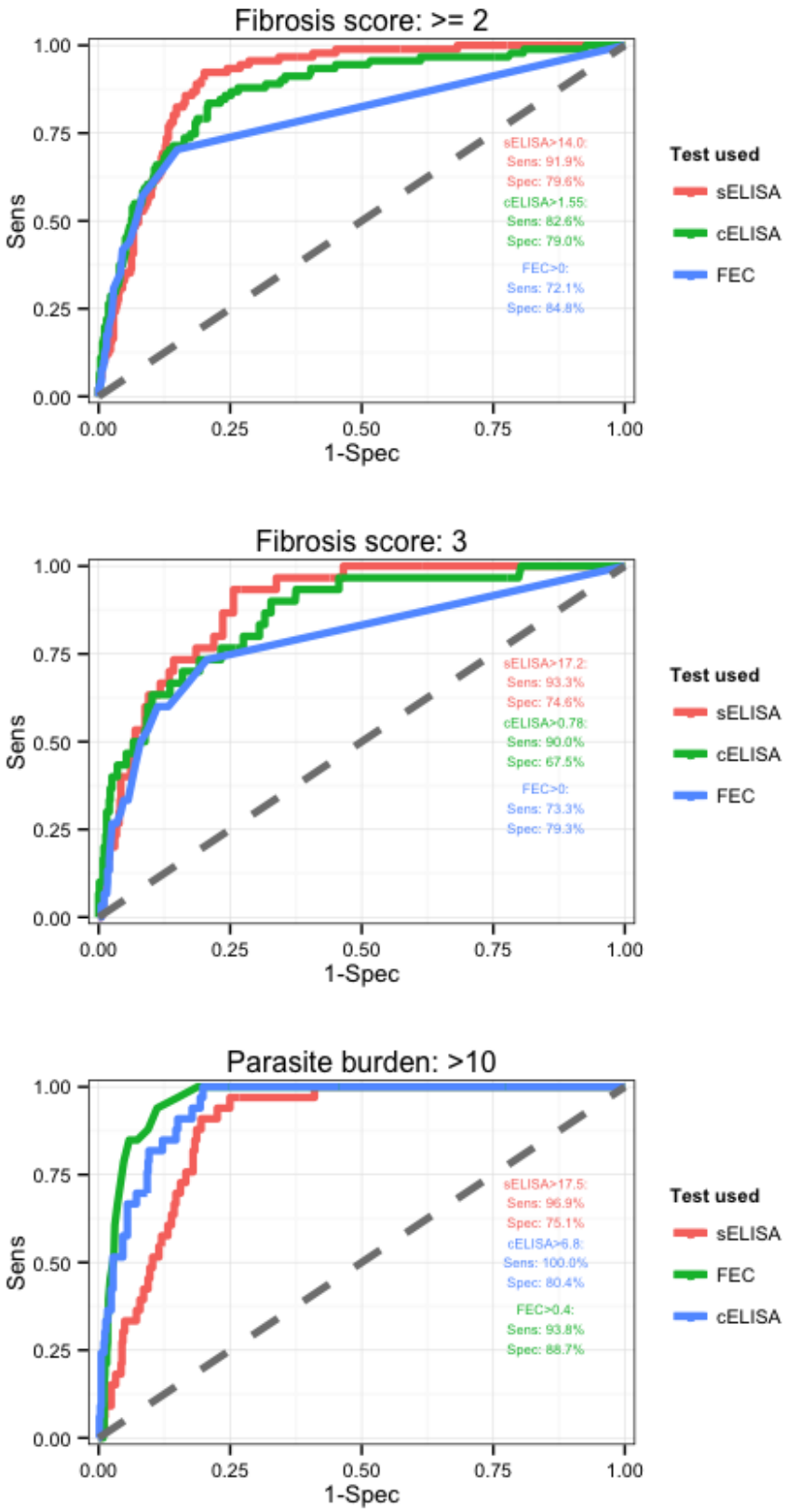


Figure 4.10: ROC curves were used to evaluate the ability of each diagnostic test to distinguish between 1) animals with a fibrosis score of 2 or more vs. a fibrosis score of 0 or 1 (top plot), 2) animals with a fibrosis score of 3 vs. a fibrosis score of 0 to 2 (middle plot), 3) animals with a parasite burden of more than 10 vs. animals with 10 or fewer flukes (bottom plot). Each plot provides suggested cut-off values for each tests along with the sensitivity and specificity estimates of each test at that cut-off value.

4.5 Discussion

The economic impact of fasciolosis in cattle is mostly through indirect, non-specific losses, caused by sub-clinical disease. It has therefore historically been difficult to demonstrate this impact and to justify spending beef farmers's limited resources towards fasciolosis control. Previous studies that have attempted to estimate the effect of fasciolosis on beef production have produced results supporting the idea that there is a negative effect on production. However, most studies were based on experimentally infected cattle, usually representing small numbers of animals, breeds or management systems, and few of the studies investigated how the effect on beef production varies depending on the severity of disease. Moreover, work by Charlier et al. (76) indicated that there is a potential for diagnostic tests to be used to inform the end-user on the extent of the damage or burden, which required further investigation.

With fasciolosis becoming a major problem in the UK cattle industry during the last decade (7; 29), estimating the effect the infection has on beef cattle has become more urgent. The aims of this study were to estimate the effect of liver fluke infection on the time taken to reach slaughter weight by beef cattle and to investigate the quantitative use of diagnostic tests in distinguishing between animals of high and low levels of infection as well as morbidity, using datasets representative of the UK beef cattle population.

Using a two year abattoir dataset we have shown that after adjusting for important covariates such as breed, sex and farm, cattle infected with liver fluke take substantially longer to reach slaughter weight when compared to uninfected cattle. More specifically, we have estimated that a beef cattle of 345 kg carcass weight took on average 10 (95% confidence interval 9-12) days longer to reach slaughter weight. Assuming that producers' decision on when to send an animal for slaughter is heavily based on

when the animals reach target weights, the difference in slaughter age we have observed is consistent with results of Sanchez-Vazquez et al. (60) who estimated that fluke infection resulted in an average slaughter weight reduction of about 0.63 kg, in the only other large scale abattoir study to investigate the effect of liver fluke infection on beef cattle production in the UK. Earlier studies reporting a negative effect of fluke on growth rates further support this result (169; 173; 176).

When considering the effect on fluke infection on growth rate using the weight and age at slaughter the question of causality becomes difficult. As there is limited development of immunity to infection, older animals have a greater chance of becoming infected at some point in their lifetime, confounding the effects of age and infection. We used a second dataset of 619 animals, sampled over three different sampling seasons, to investigate the effects of liver fluke according to severity of fibrosis. Our fibrosis and burden models showed that slaughter age increases as severity of infection increases. The fibrosis model showed that animals of mean slaugher weight of 345 kg and fibrosis score 1 took on average 34 (95% CI 11-57) days longer to reach slaughter weight, animals with fibrosis score 2 took 93 (95% CI 61-127) days longer and animals with fibrosis score 3 took 78 (95% 32-125) days longer to finish, when compared to cattle with no liver fibrosis detected. Similary, the burden model showed that when compared with animals with no liver fluke burden, animals with 1-10 parasites took on average 31 (95% CI6-56) days longer, while animals with more than 10 parasites found in their livers took 77 (95% CI31-124) days longer to finish. This dose-response relationship further supports the case of liver fluke infection having a causal relationship with lower growth rate according to basic guidelines for causality in epidemiology (190). This also indicates that it is more important to know the extent of infection, rather than solely whether the animal is infected or not.

Cattle infected with *F. hepatica* under natural conditions generally have low burden of

infection (191). It remains unclear at which level of infection production losses are induced even though cut offs have been suggested by various authors. A cut-off of 30 flukes or higher was suggested by Vercruysse et al. (191), which was challenged by Charlier et al. (76) who suggested a cut-off of 10 flukes. The burden model presented in this study supports the cut-off suggested by Charlier et al (76) as it shows that animals with a parasite burden higher than 10 take significantly longer to finish compared to uninfected animals. Furthermore, it suggests that the cutoff might lie even lower since animals with a burden of 1-10 parasites still appear to reach slaughter weight later. Nevertheless, the relationship between levels of infection and production loss is likely to be more complicated as the effect of fluke at different levels of infection is also dependent on other factors such as duration of infection, feed quality and whether animals are housed or not (191), hence making it less straightforward to use burden alone as a predictor of production loss.

Both the fibrosis and the burden models estimated a greater delay in reaching slaughter weight than the MHS model, even at the lowest levels of fibrosis and burden, respectively. While this might be counter-intuitive at the first instance, one reasonable explanation might be that there is greater misclassification of the explanatory variable measuring liver fluke infection in the MHS model. Liver inspection by the MHS has been estimated by the author to have a sensitivity of 68 % and a specificity of 88 % (chapter 3), i.e. it misclassifies 32 % of the truly positive animals and 12 % of the truly negative animals. This can result in regression dilution bias, leading the model to underestimate the effect of liver fluke infection on slaughter age (192). This can also offer an explanation as to why the MHS model does not have as good a predictive ability compared to the fibrosis and burden models. It nevertheless provides a valuable starting point, bearing in mind that the estimate is very likely to be conservative.

So far tests used for diagnosis of liver fluke infection in cattle have been used solely in a

qualitative manner, i.e. with the aim of identifying whether an animal is infected or not, but giving no indication of the extent of infection. As fasciolosis is a chronic disease in cattle causing mostly sub-clinical disease, it might be more meaningful to farmers if they know what the intensity of infection is and how that translates to production losses. This information might therefore be used to decide what treatment strategy, if any, they might decide to use. Efficient control of liver fluke is important not only to minimise production loss through currently infected animals, but also to minimise pasture contamination to prevent further infections or re-infections. Charlier et al. (92) emphasise the importance of identifying diagnostic tests, either new or existing ones, that could be used quantitatively to identify which infected animals actually require treatment and to inform producers about the extent of production loss they are suffering due to liver fluke infection.

In this context, we investigated the potential of three available diagnostic tests; FEC, coproantigen ELISA and a serum antibody ELISA, to be used quantitatively in order to improve control strategies. Positive trends were identified for the relationship between all three tests and measures of the extent of infection (burden and fibrosis score), both graphically and using Spearman rank correlation coefficients. The correlation of FECs and parasite burden is generally considered weak (70). However our study has shown that there is good positive correlation (0.79), which is similar to what was reported by a recent study by Brockwell et al. (85). We also found good correlation between the copro-antigen ELISA and parasite burden. This confirms previous such reports (83; 76; 85) where moderate to good correlation has been reported. The correlation between results of these two tests and fibrosis scores was found to be weak. Reports on the correlation between serum antibody ELISAs and parasite burden range from no correlation (193; 194; 195) to positive correlation (18; 196; 165). Our study has shown weak positive correlation (0.62) with fluke burden and moderate positive correlation (0.67) with fibrosis score.

Test result values at each level of both burden and fibrosis appear to be very variable, making it difficult to predict actual levels of infection or damage. For this reason we have used ROC curves to see how well these diagnostic tests can distinguish between cattle of high and low fluke burden using the cut-off suggested by Charlier et al. (76), and between different levels of liver damage based on fibrosis scores as presented in the second model of this study. Regarding current parasite burden, all three tests were shown to have high sensitivity at detecting animals with more than ten liver flukes (sELISA-96.9%, cELISA-100.0%, FEC-93.8%). Feacal egg counts have a high specificity of 88.7%, while the ELISA based tests have lower specificities (sELISA-75.1%, cELISA-80.4%) at the suggested cut-offs. These results indicate a potential for using the tests in practice to inform strategies on reducing both production loss and pasture contamination. In terms of fibrosis score, the two ELISA based tests appear more sensitive in picking up animals who have suffered greater damage, while having moderate specificities. In this context, tests can be used to inform producers on the extent of the damage caused by fasciolosis and can provide them with a way of monitoring and adjusting their control strategies accordingly. This is clearly an area that requires further investigation and quantitative results of these three tests can play a valuable role in the cost effective control of *F. hepatica* infection in cattle.

The results presented in this chapter highlight the importance of abattoir data in research. Availability of these data combined with with data collected by abattoir based sampling, has enabled us to fit robust models to investigate the effect of liver fluke infection on beef cattle production representative of a naturally infected population. We have presented a simple fibrosis scoring system which can be carried out within the same timeframe as routine liver inspection, which can provide producers and their vets with more informative feedback on the extend of the problem of fasciolosis at a particular farm, allowing better monitoring of control strategies and evaluation of potential losses due to liver fluke. Further work is planned to validate this model at the

abattoir for a longer period of time, which can then provide a robust system that can be adapted at various abattoirs in order to provide producers with more efficient abattoir feedback.

Overall, this study has provided robust evidence that cattle infected with liver fluke take substantially longer to reach slaughter weight compared with non infected animals and this depends on the extent of infection. Additionally, we have provided further evidence to support that available diagnostic tests correlate with measures of infection or morbidity and can be used to identify animals with high burdens or high degree of fibrosis and hence improve the efficiency of control strategies. Lastly, we suggest fibrosis score methodology to be routinely used at slaughterhouses in order to provide more informative liver inspection results, which can further inform and improve currently adopted control strategies.

Chapter 5

Risk factor analysis for liver rejections due to signs of F. hepatica infection in cattle slaughtered in Scotland

5.1 Abstract

Fasciolosis, a ruminant disease of worldwide distribution caused by the trematode parasite *Fasciola hepatica*, has been shown to cause important production losses on beef cattle production. In the UK, there has been an increase in both the incidence of fasciolosis and resistance to triclabendazole, one of the most important anthelmintics available for the treatment of the disease. At the same time, models based on predicted weather data estimate that the risk of infection will continue to increase. Practical guidelines on sustainable fluke management are therefore urgently required. The aim of this study was to describe the proportion of livers rejected due to signs of liver fluke

in cattle slaughtered at a large red meat abattoir in Scotland and identify risk factors associated with these rejections. Various data sources were used for this analyis: a) animal level data from an abattoir dataset of cattle slaughtered during 2013 and 2014 consigned from Scottish holdings, b) data collected using a herd management questionnaire and c) climatic and environmental data. A multi-variable mixed effects logistic regression model was built to estimate the association between climate, environmental, management and animal specific factors and the risk of an animal being infected by *F. hepatica*. Multiple imputation methodology was employed to deal with missing data arising from skipped questions in the questionnaire. More than one in four livers were rejected due to signs of liver fluke in the 112,127 cattle consigned from Scottish holdings slaughtered during the two year period. The percentage of livers rejected reached 30% in 2013 and 20% in 2014. Results of the regression model confirmed the importance of temperature, rainfall and cattle movements in increasing the risk for fasciolosis, while it indicated that the presence of deer can increase the risk of infection and that male cattle have a reduced risk of infection. As weather conditions remain favourable for fluke transmission, the number of cattle movements remains high and emergence of anthelmintic resistance continues, it is extremely important to be able to provide practical guidelines for sustainable ways to control liver fluke. The model failed to identify specific management factors which affect the risk of infection. This is likely to be because one solution does not fit all when dealing with liver fluke infection, although climate factors will always play an important role. Future work should focus on more detailed on farm studies in order to identify finer scale indicators of fasciolosis risk, specific to different farm settings.

5.2 Introduction

Fasciolosis in the UK was traditionally a disease of the wetter western regions arising from summer infection of snails and resulting in acute disease in autumn followed by chronic disease later on. During the last decade or so, the incidence of fasciolosis in cattle and sheep has been described to be increasing. Furthermore the infection has been reported in areas and farms that were previously considered to have low fasciolosis risks, such as those in the East of Scotland, causing major losses, where farmers have been unprepared. The timing of the infection appears to be more variable, with acute infections reported earlier than before. This can be explained by the fact that milder and wetter weather conditions permit overwintering of infected snails as well as being conducive to prolonged survival of infective metacercariae on pasture (47).

The epidemiology of liver fluke in the UK is clearly changing and the described changes in incidence, seasonality and geographical distribution have been repeatedly attributed to climate change, increasing animal movements and development of anthelmintic resistance (48; 43). Resistance to triclabendazole, the only flukicide able to kill early-immature stages of infection, is now thought to be a problem in various parts of the world and its increase is expected to further comproromise fluke control. Similarly, resistance has been reported in other flukicidal drugs such as cloasantel and albendazole (99). At the same time there are no commercially available vaccines against liver fluke, and to date no experimental cattle vaccines have shown adequate efficacies (110).

Studies by Fox et al. (17) and Caminade et al. (49) have modelled the future risk of *Fasciola hepatica* infection based on predicted weather data. In the UK, and generally in northern Europe, fasciolosis incidence is predicted to continue to increase, while the

season suitable for parasite development outside the mammalian host is expected to be extended. This predicted increase in incidence, along with the increase in constraints to fluke control for the reasons stated above, makes this a crucial time to be able to provide applicable guidelines on sustainable fluke management.

Transmission of *F. hepatica* is known to be highly dependent on temperature (average daily temperatures of more than 10^{o}C) and high moisture levels, which are relevant for both optimum conditions for parasite egg development as well as for reproduction and survival of its intermediate host, commonly *Galba truncatula* in the UK (15; 5; 16). For this reason, predictive models using climate data have been available for a long time (15; 32). McCann et al. (53; 54) emphasised that even though the distribution of *F. hepatica* in England and Wales can be explained by climatic and environmental factors, the great variability between closely located regions sharing climatic characteristics is possibly explained by differing management strategies employed. Nevertheless, there are only a few studies investigating the importance of management strategies, which have mainly focused on dairy cattle.

Charlier et al. (52) combined management questionnaire information with on site investigations in order to identify and characterize possible snail habitats and investigate the presence of snails. The study included 39 dairy herds in Belgium and identified the number of potential snail habitats, the presence of snails, pasture drainage, the month cows were turned out, stocking rate and type of watering place as risk factors associated with *F. hepatica* infection, measured by indirect bulk tank milk ELISA. In another study in Belgium on 1762 dairy herds (51) the factors associated with liver fluke infection included annual rainfall, pasture mowing, increased proportion of grazed grass in the diet and longer grazing season. Length of the grazing period was also identified as a risk factor in a study in dairy cattle in Sweden (145).

Using antibody ELISA results on bulk milk tank samples from 606 herds from Eng-

land, Wales and Scotland, Howell et al. (197) found that higher rainfall, grazing boggy pasture, presence of beef cattle on farm, access to a permanent water source and smaller herd size were associated with higher risk of *F. hepatica* infection. To the author's knowledge there are no UK studies investigating risk factors for *F. hepatica* infection in beef cattle, taking into consideration climatic, environmental and management factors.

The current study aims to fill this gap using a two year abattoir dataset, data from a herd management questionnaire, climatic and environmental data to identify risk factors for liver rejection due to signs of *F. hepatica* infection in cattle slaughtered at a large red meat abattoir in Scotland.

5.3 Materials and methods

5.3.1 Data Sources

Animal level data

Animals included in this study were cattle slaughtered at the Scotbeef Limited abattoir in Scotland during 2013 and 2014 consigned from Scottish holdings. Scotbeef abattoir is located in central Scotland, and even though it receives animals from the whole of the UK, a great proportion of its producers come from Scotland which is where this study focuses on. The liver fluke status of each animal was determined by the results of liver inspection carried out by the Meat Hygiene Service. Details on the routine collection of data at Scotbeef abattoir can be found in chapter 2.

Climatic and environmental data

Soil type and pH data were sourced from the National Soil Map of Scotland which was created by the Macaulay Land Research Institute using data collected between 1947 and 1981 (134). UK Climate Impacts Programme (UKCIP) 5 km grid mean monthly data for rainfall and temperature between 2007-2011 were obtained from the UK Meteorological Office (135; 136). These were first averaged per 3 month periods (February-March-April, May-June-July, August-September-October, November-December-January) and then averaged over five years as suggested by McCann et al. (54). River data were extracted from the OS Open Rivers dataset sourced from EDINA (137) and distance to the closer river was calculated for each holding of origin. Slope and elevation data were extracted from the OS terrain 50 digital terrain model (DTM) data, downloaded from EDINA (138). Farm area, as reported in the questionnaire, was used to construct a circle around the geographic coordinates of each farm, for which a mean slope and elevation was calculated using the 50m DTM. R packages used for the extraction of the climate and environmental data for each farm included *raster* (198), *SDMTools* (199), *rgeos* (200), *rtiff* (201) and *maptools* (202).

Farm management questionnaire

Information about the type of farm and management strategies used at each farm was collected through an online questionnaire. The questionnaire contained questions about the following: a) general farm details, b) respondent's details, c) type and numbers of animals kept, d) management of bought in animals, e) presence of other animals other than cattle on the farm, f) grazing management, g) pasture management, h) liver fluke management, and i) liver fluke history. The complete questionnaire can be found in Appendix A. Details about the pilot and administration of the questionnaire can be found in chapter 2.

5.3.2 Statistical Analysis

Exploratory descriptive analysis and mixed-effects logistic regression

Statistical analysis was carried out using R (Version 3.0.3) (155) within RStudio (Version 0.98.1091) (203). A multi-variable mixed effects logistic regression model was built to estimate the association between climate, environmental, management and animal specific factors and the risk of an animal being infected by *F. hepatica* using package *lme4* (181). Animals with livers rejected due to signs of liver fluke infection were classified as infected for the purposes of this analysis. As the risk of being infected by *F. hepatica* for animals coming from the same producer could not be assumed to be independent, producer was introduced in the model as a random effect. In other words, the outcome for the model was individual animal (i) liver rejection due to signs of liver fluke infection with producer (j) used as a random effect as shown below:

$$y_{ij} \sim \alpha + \beta X_{ij} + \mu_j + \varepsilon_{ij}$$

Where α is the fixed intercept, β the fixed effects, X the covariates, μ_j the random effects, ε_{ij} the error, $\mu \sim N(0, \sigma^2_{herd})$ and $\varepsilon \sim N(0, \sigma^2_{animal})$.

Data checking was first carried out in order to identify missing values, categories with very few values or values outside expected ranges. Frequency tables were constructed for binary and categorical variables and summary statistics were obtained for continuous variables. Logistic regression modelling has a strong assumption of a linear relationship between numeric explanatory variables and the log odds of the response variable (192). The relationship between all numerical variables and the outcome was investigated by visualisation using univariable smoothed scatterplots on the logit scale (204) and by using the Box-Tindwell test (205). This involves including an interaction term between each variable and its natural log in each univariable model and rejecting the assumption of linearity if this term is significant. Variables were then either

categorised or transformed as necessary. Additionally, correlation between pairs of numerical variables was investigated by drawing a correlation plot with Pearson correlation coefficients using package *corrplot* (157). In cases where high correlations between pairs of variables was detected one variable from each pair was included in the multivariable analysis.

Missing data

Missing data (MD) is a commonly encountered problem in veterinary epidemiological studies carrying out risk factor analysis. Missing data can arise in various ways and can be of three main types; “Missing completely at random" (MCAR), “missing at random" (MAR) and “missing not at random" (MNAR) (206). MCAR values have no systematic differences to observed values, for example when a paper copy of a questionnaire falls in a puddle and some entries are no longer legible or when labels of a tray of samples come off. On the other hand in both “missing at random" and “missing not at random" data systematic differences exist between observed and missing values. In the case of values MAR observed data can be used to explain such differences, while in values MNAR that is not possible. For example the age of an animal may be missing more frequently in farmers who do not keep records for each animal in comparison with those who do. If information about record keeping is available then missing data can be considered MAR, while if no such information has been collected then the missing values are MNAR as not enough information is collected to explain the systematic differences between observed and missing values. In reality it is very difficult to distinguish between values MAR and values MCAR, but inclusion of as many predictors as possible in the imputation model makes it more plausible that the probability of missingness can be explained by the observed data (207).

There are numerous methods for dealing with missing data of varying degrees of diffi-

culty and validity split into *ad hoc* or principled methods. Easy to use, *ad hoc* methods are commonly used even though they have not been shown to be statistically valid (206). These include complete case analysis (CCA), missing indicator method (MIM), overall mean/mode substitution (OMS) and last observation carried forward (LOCF). A great variety of principled methods for dealing with MD have been around for a long time and include single or multiple imputation, likelihood or Bayesian based approaches and more. In general, such methods use available information to generate information about the missing values or the missing value mechanism, while never replacing missing values directly. The major difference between principled and ad hoc methods is that the former can be statistically valid under explicit assumptions about the mechanism of missing data (www.missingdata.org.uk).

Multiple imputation methods use a model to predict the distribution of the missing data based on that of the observed data, thus creating multiple complete datasets. In each of the imputed datasets, observed data remain the same, while missing data are replaced with a different value each time. In this way, the imprecision arising due to the fact that the distribution of the missing values is estimated, is accounted for, which leads to more accurate results by not underestimating the standard errors. Each imputed dataset is then analysed using standard statistical techniques and the results are averaged to provide combined estimates. Such imputation techniques can only be considered unbiased if the missing values are missing at random or missing completely at random, as explained above (208; 209).

For the purposes of this analysis, five imputed datasets containing information on management, climate and environment were created at the producer level using multiple imputation package *Amelia* (210). *Amelia* assumes that the data are missing at random so it predicts the missing values based on the information provided by the other variables in the dataset (208). Each dataset was then merged with data at the animal level

from the abattoir dataset using package *plyr* (211). For the purposes of model building and validation, each dataset was split into a training set (70% of producers) and a test set (30% of the producers). Univariable analysis was carried out using packages *Zelig* (212) and *ZeligMultilevel* (213) providing combined results.

Model selection

Model selection was carried out on each of the five imputed datasets separately. Variables with a p-value lower than 0.2 in the univariable analysis (204) were considered for the final model using manual backward elimination. In other words, all variables were included in the initial model and then removed one by one sequentially (214). Akaike information criterion (AIC) (215; 216) was used to judge whether a variable would remain or be removed from the model. AIC penalises the log likelihood of each model according to the number of parameters in order to identify the most parsimonious model (217; 218). Therefore, if removal of a variable decreased the AIC then the variable was removed, while if the AIC remained the same or was increased then the variable was retained in the model. Variables retained in all five models were kept in the final model. Combined AIC was used to decide whether to keep variables kept in four or three out of the five models. Combined AIC refers to the average of the AIC from the models run on each of the five imputed datasets.

Model assessment

In order to assess the presence of spatial autocorrelation in the residuals of the final model, an empirical semi-variogram was plotted and visually inspected using package *gstat* (219). In other words, we plotted a scatterplot of half the square of the difference (semi-variance) in residuals between each pair combination of locations where animals originated from, against the distance between them (220). If positive spatial correlation

exists, one would expect that semi-variance between locations closer to each other is smaller and that it increases as distance between locations increases. Thereofore, a straight line would indicate no spatial autocorrelation, since variability in the residuals is not explained by distance between locations (221; 222). Sensitivity analysis was carried out in order to check whether using multiple imputation affected the direction of effect of each variable on the outcome variable. This was achieved by running the same model on the training dataset using listwise deletion and on the imputed test datasets and plotting the effects of each variable in each model. The predictive ability of the final model was assessed using area under the curve (AUC) of the receiver operating characteristic (ROC) curve using the *pROC* (223) package, both assessing how well the model would predict the outcome variable in the training dataset as well as the test dataset. Package *ggplot2* (158) was used to draw the plots presented in this work.

5.4 Results

5.4.1 Sample Demographics

In total, 112,127 cattle between 365 and 2,000 days old were sent to the abattoir between 2013 and 2014 from 1,106 Scottish based producers. Producers submitted on average 9 cattle on a single day, ranging from 1 to 80. Producers submitted on average 63 animals per year ranging from 1 to 1,603. In 2013 the mean proportion of livers rejected due to liver fluke per producer was 0.29 (95 % Confidence Interval (CI) 0.28-0.30), while in 2014 it dropped to 0.20 (95 % CI 0.19-0.22). Figure 5.1 shows the geographical distribution of the producers and the proportion of animals with livers rejected due to signs of liver fluke per producer for these two years. Furthermore, Figure 5.2, shows the proportion of livers rejected due to liver fluke per month comparing trends for 2013 and 2014. It is apparent that levels of rejections of livers due to signs of liver fluke were higher in 2013.

The questionnaire was completed by 214 Scottish based producers sending 42,521 cattle to the abattoir. As can be seen from Figure 5.2 the proportions of livers rejected per month in this subset are similar to the overall figures. In fact the average proportion of livers rejected due to liver fluke per producer in 2013 was 0.31 (95% CI 0.29-0.34), while in 2014 it was 0.17 (95% CI 0.16-0.19) which is similar to the overall figure. Additionally, the geographical distribution of producers who completed the questionnaire is comparable to that of all the producers. Data from 150 of those producers (27,048 cattle) were used to create the training dataset, while data from 64 producers (15,473 cattle) were retained as a validation dataset.

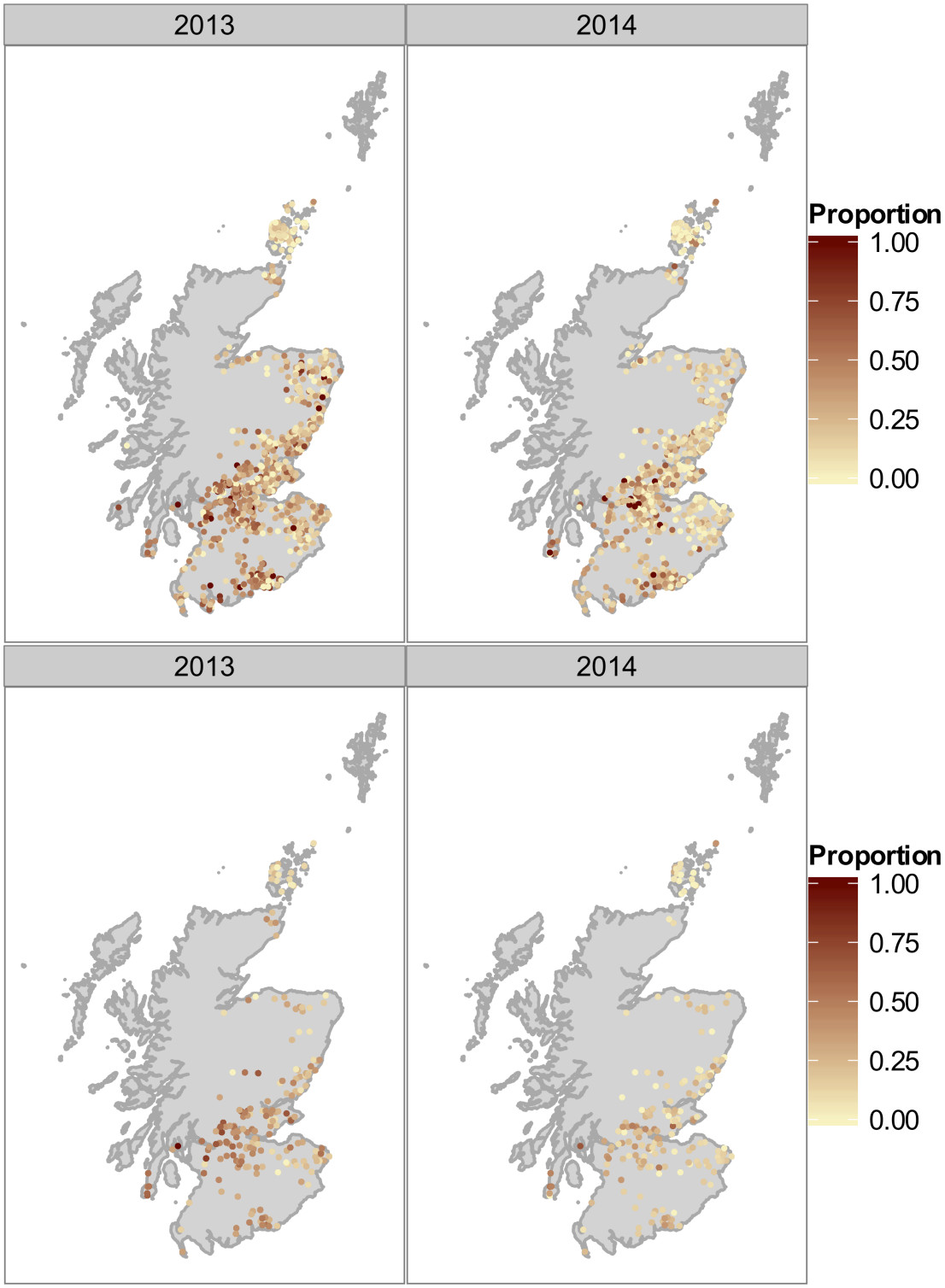


Figure 5.1: Geographical distribution of farms with proportions of liver fluke rejections. Figure shows the proportion of livers rejected due to signs of liver fluke infection by each producer and their geographical location. Top plots refers to all Scottish based producers, while bottom plots refers to producers who completed the questionnaire.

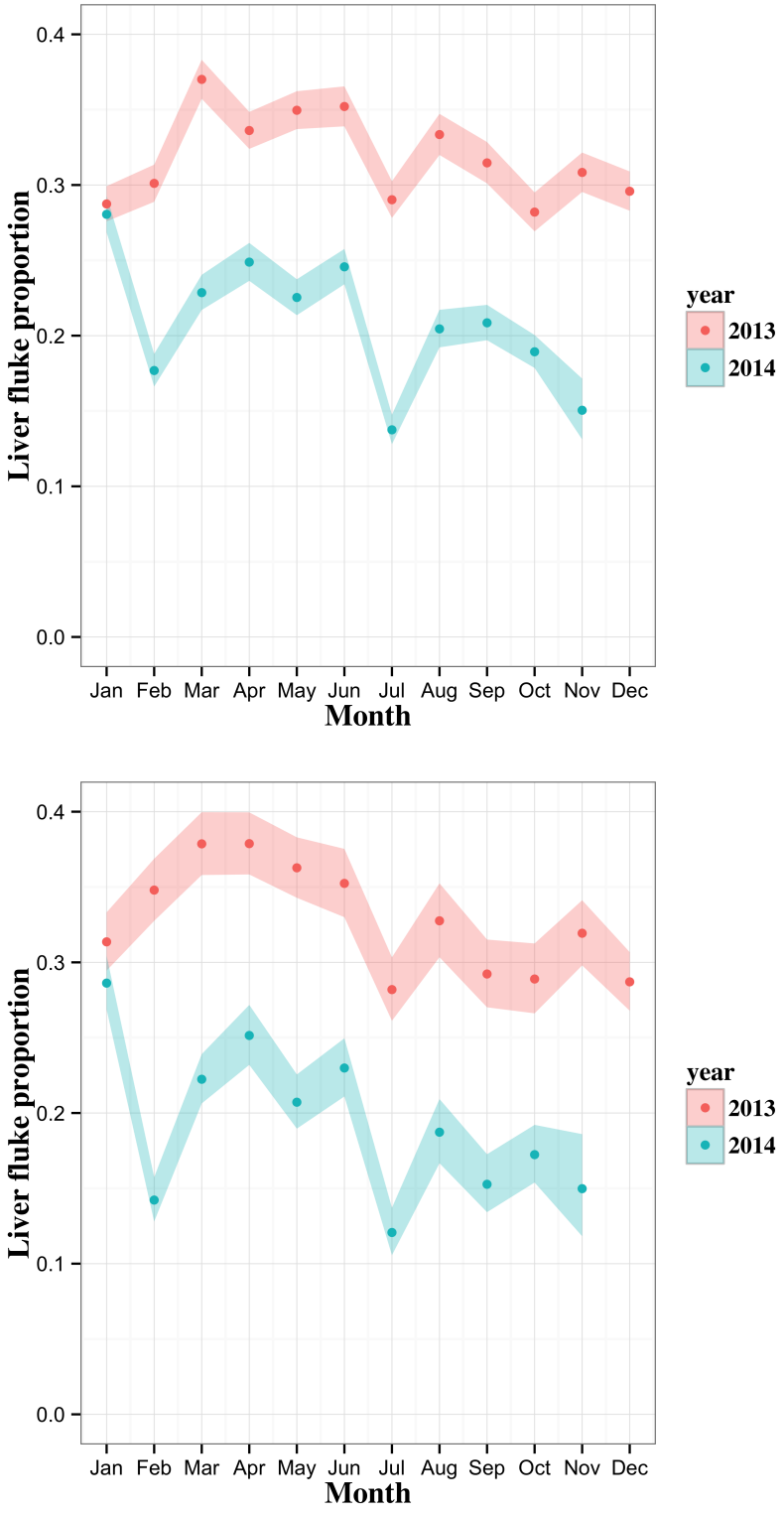


Figure 5.2: Monthly proportions of liver rejections due to liver fluke. Figure shows the monthly proportions of livers rejected due to signs of liver fluke infection in 2013 and 2014. Top plot shows the results for all Scottish based cattle producers, while bottom plot shows only the producers who competed the questionnaire. Trends are comparable between the two plots and 2013 is consistently a more severe year in terms of liver rejections.

5.4.2 Missing values

Most of the questions in the dataset (variables) were skipped by one or more producers, with variables containing up to 0.32 missing values. This can be seen in Figure 5.3 (top), which shows the proportion of missing values in each variable at the producer level. The location of missingness by variable and producer is shown in Figure 5.3 (bottom).

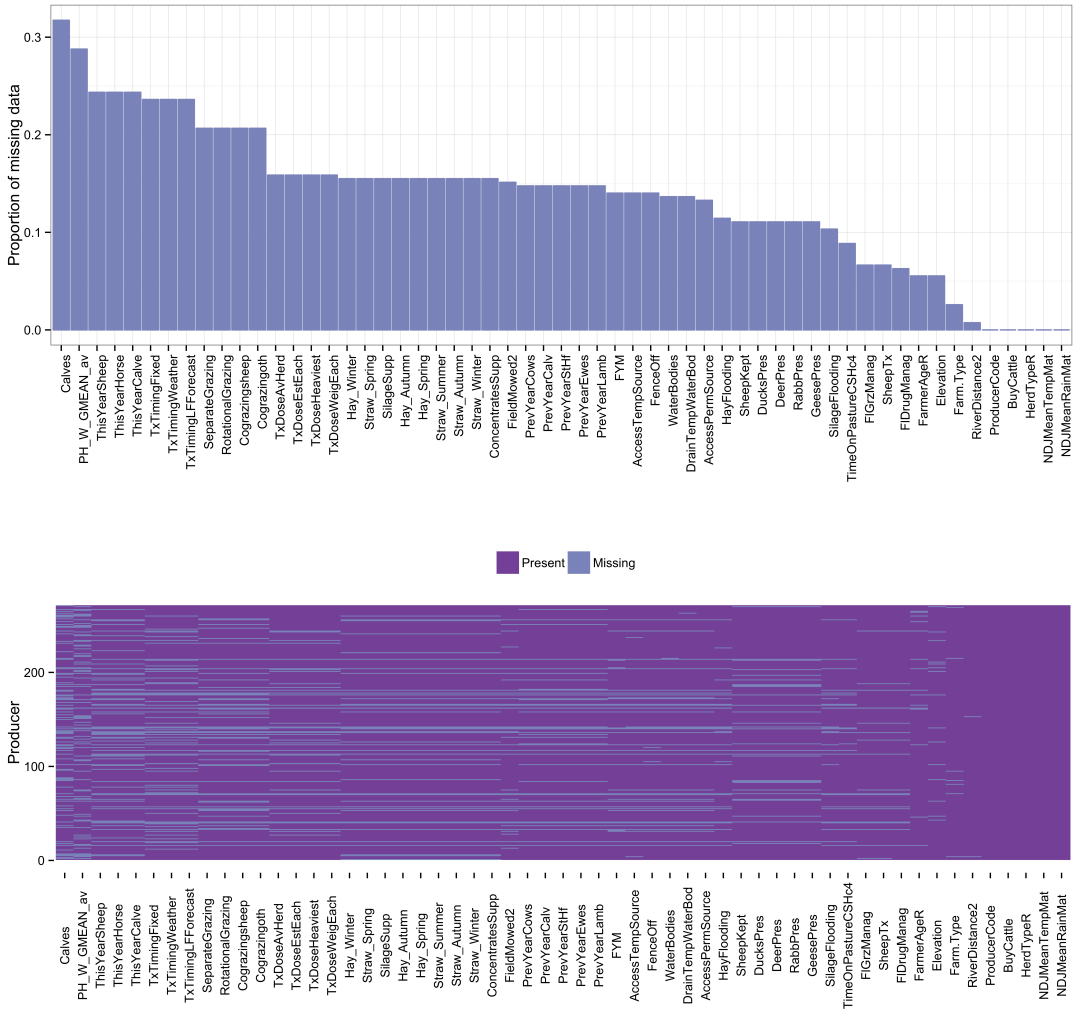


Figure 5.3: Distribution and proportion of missing values per variable. Top plot shows the proportion of missing data in each variable, while bottom plot shows the distribution of missingness in each variable according to each producer.

5.4.3 Variable summary and univariable analysis

Summary tables or statistics for each variable are included in Table 5.1 and Table 5.2 for numerical and categorical variables respectively. These tables also include an explanation for which question the name of each variable refers to. Results of the univariable analysis are shown in Table D.1 in appendix D.

Table 5.1: Summary values for numerical variables. Table contains explanation for each variable name to be used in the rest of the tables and plots. All data shown here were sourced from climatic and environmental data sources described above except *age_days*, which was sourced from routine abattoir data and *Calves*, which was sourced from Q.13 in the questionnaire.

Variable name	**Description**	**Min.**	**Median**	**Mean**	**Max.**
PH_W_GMEAN_av	Soil pH	3.64	4.90	4.81	7.211
Elevation	Elevation	4.46	79.20	92.68	254.80
Slope	Slope	0.005	0.06	0.06	0.18
NDJMeanTempMat	Mean Temperature (Nov-Dec-Jan)	1.85	3.99	3.99	6.17
FMAMeanTempMat	Mean Temperature (Feb-Mar-Apr)	3.19	5.30	5.27	6.26
MJJMeanTempMat	Mean Temperature (May-Jun-Jul)	10.41	12.22	12.24	13.46
ASOMeanTempMat	Mean Temperature (Aug-Sep-Oct)	9.18	11.28	11.25	12.19
NDJMeanRainMat	Mean Rainfall (Nov-Dec-Jan)	59.44	97.10	103.80	206.40
FMAMeanRainMat	Mean Rainfall (Feb-Mar-Apr)	33.34	54.92	59.52	103.20
MJJMeanRainMat	Mean Rainfall (May-Jun-Jul)	42.42	59.59	61.17	88.72
ASOMeanRainMat	Mean Rainfall (Aug-Sep-Oct)	58.82	94.49	103.00	192.70
age_days	Cattle age in days	366	694.00	691.00	1199.00
Calves	Number of calves kept	0	100.00	190.10	750

5.4.4 Multivariable analysis

Variables with a p-value of less than 0.2 were considered for inclusion in the final model and are shown in Table 5.3. The variable selection procedure based on AIC values for each of the five imputed datasets is shown in Table 5.4. Variables remaining in each of the 5 imputed datasets based on the model selection procedure described are shown in Table 5.5. Variables "BuyCattle", "DeerPres", "Straw Spring", "season", "year", "MJJMeanTempMat", "MJJMeanRainMat" were retained in all five models so they were kept in the final model. Combined AIC was used to decide whether to keep the variables kept in four or three out of the five models and this resulted in also keeping variables "age days", "FlDrugManag", "sex" and "PH W GMEAN av".

The final model is shown in Table 5.6. It shows that a male animal has a decreased risk of having a liver rejected due to signs of liver fluke infection with an Odds Ratio (OR) of 0.94 (95% Confidence Interval (CI) of 0.88-1). Straw supplementation during spring also seems to have a protective effect against liver fluke infection (OR 0.79, 95% CI 0.64-0.97). Furthermore, animals being slaughtered during the 2nd, 3rd and 4th quarter of the year appear to have a lower risk of having livers rejected due to liver fluke: Q2 OR 0.94 (95% CI 0.87-1.01), Q3 OR 0.73 (95% CI 0.67-0.80), and Q4 OR 0.71 (95% CI 0.65-0.78). Lastly, an animal slaughtered in 2014 had a decreased risk with an OR of 0.45 (95% CI 0.45-0.46).

On the other hand, animals had an increased risk of rejection if they originated from farms where cattle are bought in (OR 1.31, 95% CI 1.05-1.64), where deer were present (OR 1.18, 95% 0.96-1.46), farm that had higher mean temperatures (OR 1.44, 95% CI 1.22-1.69) and higher mean rainfall (OR 1.02, 95% CI 1.01-1.03) as well as greater soil pH (OR 1.15, 95% CI 0.94-1.42) and farms where flukicides were used to manage the risk of liver fluke (OR 1.30, 95 % 1.01-1.68). Lastly, the risk of an animal having its liver rejected due to signs of liver fluke increased by age at slaughter (OR 1.001,

95% CI 1.001-1.002).

The AUC calculated to assess the predictive ability of this final model, i.e. to assess how good the model is in predicting the outcome variable, in the training dataset as well as the validation dataset were 0.72 and 0.63 respectively. Figure 5.4 shows how the estimates from this model compare with the estimates of the model being fitted on the train dataset using listwise deletion and on the imputed validation datasets. The results of the final model are very comparable with the listwise model, showing that the use of multiple imputation did not introduce bias to the results. Nevertheless, there are greater differences between the estimates from the final model and that of the validation model partly explaining why the predictive ability of the model is worse when predicting the outcome of the validation dataset. Lastly, variograms in Figure 5.5 show that there was no strong evidence of spatial autocorrelation.

Table 5.2: Tables of main questionnaire results. Table contains explanation for each variable name to be used in the rest of the tables and plots. Information in brackets indicate the questionnaire question number data were sourced from unless otherwise indicated.

Variable	Description	Number	Proportion
FarmerAgeR	Producer's age (Q.6)		
<30		9	0.04
30-49		98	0.48
>49		96	0.47
Missing		11	0.05
HerdTypeR	Type of herd (Q.8)		
Cattle finisher		68	0.32
Cattle breeder/finisher		135	0.63
Cattle breeder		11	0.05
Missing		0	0.00
Farm.Type	Type of farm (abattoir data)		
Hill		34	0.16
Low Ground Arable		103	0.49
Marginal		72	0.34
Missing		5	0.02
BuyCattle	Producer buys cattle (Q.14)		
No		68	0.32
Yes		146	0.68
Missing		0	0.00
BuyCattleHowOften	How often they buy cattle (Q.15)		
No		60	0.28
Less often than once per year		26	0.12
Annually		38	0.18
More often than once per year		88	0.42
Missing		2	0.01
NewCatDeworm	Deworm new cattle (Q.18)		
No		34	0.17
Yes		115	0.56
Don't Buy		57	0.28
Missing		8	0.04
NewCatIsol	Isolate new cattle (Q.20)		
No		59	0.29
Yes		81	0.39
Don't Buy		55	0.27
Not applicable		11	0.05
Missing		8	0.04
SheepKept	Producer keeps sheep (Q.22a)		
No		46	0.24
Yes		143	0.76
Missing		25	0.12
GoatsKept	Goats kept on farm (Q.22b)		
No		188	0.99
Yes		1	0.01
Missing		25	0.12
DucksKept	Ducks kept on farm (Q.22c)		
No		183	0.97
Yes		6	0.03
Missing		25	0.12

Table 5.2 continued - part 1.

Variable	Description	Number	Proportion
GeeseKept	Geese kept on farm (Q.22d)		
No		183	0.97
Yes		6	0.03
Missing		25	0.12
DucksPres	Ducks present on farm (Q.22e)		
No		136	0.72
Yes		53	0.28
Missing		25	0.12
GeesePres	Geese present on farm (Q.22f)		
No		114	0.60
Yes		75	0.40
Missing		25	0.12
DeerPres	Deer present on farm (Q.22g)		
No		67	0.35
Yes		122	0.65
Missing		25	0.12
RabbPres	Rabbits present on farm (Q.22h)		
No		56	0.30
Yes		133	0.70
Missing		25	0.12
FYM	Farm yard manure used (Q.32a)		
No		101	0.55
Yes		84	0.45
Missing		29	0.14
TimeOnPastureCSH	Months spent on pasture (calves, heifers, steers) (extracted from Q.23)		
0 - 4 months		32	0.16
5 - 8 months		155	0.79
9 - 12 months		9	0.05
Missing		18	0.08
PrevYearCows	This year's cattle grazing pasture used by cows last year (Q.26a)		
No		57	0.31
Yes		127	0.69
Missing		30	0.14
PrevYearCalv	This year's cattle grazing pasture used by calves last year (Q.26b)		
No		50	0.27
Yes		134	0.73
Missing		30	0.14
PrevYearStHf	This year's cattle grazing pasture used by steers/heifers last year (Q.26c)		
No		23	0.12
Yes		161	0.88
Missing		30	0.14
PrevYearEwes	This year's cattle grazing pasture used by ewes last year (Q.26d)		
No		85	0.46
Yes		99	0.54
Missing		30	0.14

Table 5.2 continued - part 2.

Variable	Description	Number	Proportion
PrevYearLamb	This year's cattle grazing pasture used by lambs last year (Q.26e)		
No		84	0.46
Yes		100	0.54
Missing		30	0.14
ThisYearSheep	This year's cattle grazing pasture also used by sheep (Q.27a)		
No		40	0.24
Yes		124	0.76
Missing		50	0.23
ThisYearHorse	This year's cattle grazing pasture also used by horses (Q.27b)		
No		153	0.93
Yes		11	0.07
Missing		50	0.23
ThisYearCalve	This year's cattle grazing pasture also used by calves (Q.27c)		
No		65	0.40
Yes		99	0.60
Missing		50	0.23
SeparateGrazing	Each species grazes on separate field (Q.28a)		
No		99	0.59
Yes		70	0.41
Missing		45	0.21
RotationalGrazing	Rotational grazing used (Q.28b)		
No		78	0.46
Yes		91	0.54
Missing		45	0.21
Cograzingsheep	Cattle cograze with sheep (Q.28c)		
No		102	0.60
Yes		67	0.40
Missing		45	0.21
Cograzingoth	Cattle cograze with other animals (Q.28d)		
No		167	0.99
Yes		2	0.01
Missing		45	0.21
Hay_Autumn	Hay supplemented during autumn (Q.29)		
No		163	0.88
Yes		22	0.12
Missing		29	0.14
Hay_Winter	Hay supplemented during winter (Q.29)		
No		145	0.78
Yes		40	0.22
Missing		29	0.14
Hay_Summer	Hay supplemented during summer (Q.29)		
No		184	0.99
Yes		1	0.01
Missing		29	0.14
Hay_Spring	Hay supplemented during spring (Q.29)		
No		159	0.86
Yes		26	0.14
Missing		29	0.14

Table 5.2 continued - part 3.

Variable	Description	Number	Proportion
Straw_Autumn	Straw supplemented during autumn (Q.29)		
No		98	0.53
Yes		87	0.47
Missing		29	0.14
Straw_Winter	Straw supplemented during winter (Q.29)		
No		57	0.31
Yes		128	0.69
Missing		29	0.14
Straw_Spring	Straw supplemented during spring (Q.29)		
No		101	0.55
Yes		84	0.45
Missing		29	0.14
Straw_Summer	Straw supplemented during summer (Q.29)		
No		165	0.89
Yes		20	0.11
Missing		29	0.14
Silage_Autumn	Silage supplemented during autumn (Q.29)		
No		87	0.47
Yes		98	0.53
Missing		29	0.14
Silage_Winter	Silage supplemented during winter (Q.29)		
No		31	0.17
Yes		154	0.83
Missing		29	0.14
Silage_Spring	Silage supplemented during spring (Q.29)		
No		69	0.37
Yes		116	0.63
Missing		29	0.14
Silage_Summer	Silage supplemented during summer (Q.29)		
No		175	0.95
Yes		10	0.05
Missing		29	0.14
Concentrates_Autumn	Concentrates supplemented during autumn (Q.29)		
No		51	0.28
Yes		134	0.72
Missing		29	0.14
Concentrates_Winter	Concentrates supplemented during winter (Q.29)		
No		35	0.19
Yes		150	0.81
Missing		29	0.14
Concentrates_Spring	Concentrates supplemented during spring (Q.29)		
No		63	0.34
Yes		122	0.66
Missing		29	0.14
Concentrates_Summer	Silage supplemented during summer (Q.29)		
No		131	0.71
Yes		54	0.29
Missing		29	0.14

Table 5.2 continued - part 4.

Variable	Description	Number	Proportion
HayFlooding	Hay pasture suffered flooding (Q.30)		
No		173	0.91
Yes		17	0.09
Missing		24	0.11
SilageFlooding	Silage pasture suffered flooding (Q.31)		
No		161	0.83
Yes		32	0.17
Missing		21	0.10
WaterBodies	Farm near water bodies (Q.32b)		
No		89	0.48
Yes		96	0.52
Missing		29	0.14
FenceOff	Water bodies fenced off (Q.32c)		
No		122	0.66
Yes		62	0.34
Missing		30	0.14
DrainTempWaterBod	Temporary water bodies drained (Q.32d)		
No		102	0.55
Yes		83	0.45
Missing		29	0.14
AccessTempSource	Animals have access to temporary water sources (Q.32e)		
No		100	0.54
Yes		85	0.46
Missing		29	0.14
AccessPermSource	Animals have access to permanent water sources (Q.32f)		
No		116	0.62
Yes		70	0.38
Missing		28	0.13
FieldMowed2	Percentage of fields mowed (Q.34)		
No		77	0.43
Yes $<$ 50% of fields		62	0.34
Yes $>$ 50% of fields		41	0.23
Missing		34	0.16
FlGrzManag	Grazing management used to reduce fluke risk (Q.38b)		
No		130	0.65
Yes		69	0.35
Missing		15	0.07
FlDrugManag	Flukicides used (Q.38c)		
No		39	0.20
Yes		160	0.80
Missing		15	0.07

Table 5.2 continued - part 5.

Variable	Description	Number	Proportion
TxDoseEstEach	Animals treated by estimating each animal's weight (Q.40a)		
No		90	0.50
Yes		91	0.50
Missing		33	0.15
TxDoseAvHerd	Animals treated using average herd weight (Q.40b)		
No		165	0.91
Yes		16	0.09
Missing		33	0.15
TxDoseHeaviest	Animals treated using heaviest animal's (Q.40c) weight (Q.40a)		
No		164	0.91
Yes		17	0.09
Missing		33	0.15
TxDoseWeigEach	Animals treated by weighing each animal (Q.40d)		
No		128	0.71
Yes		53	0.29
Missing		33	0.15
TxTimingFixed	Treatment timing is fixed (Q.41a)		
No		63	0.39
Yes		98	0.61
Missing		53	0.25
TxTimingWeather	Treatment timing depends on weather (Q.41b)		
No		113	0.70
Yes		48	0.30
Missing		53	0.25
TxTimingLFForecast	Treatment timing informed by liver fluke forecast (Q.41c)		
No		142	0.88
Yes		19	0.12
Missing		53	0.25
SheepTx	Sheep treated for liver fluke (Q.42)		
No		28	0.14
No sheep kept on farm		61	0.31
Yes		110	0.55
Missing		15	0.07
LfFEC	Monitor Faecal Egg Counts (Q.43g)		
No		145	0.79
Yes		38	0.21
Missing		31	0.14
RiverDistance2	Distance to the nearest river (EDINA data)		
<=500 m		135	0.63
>500 m		79	0.37
Missing		0	0.00
sex	Animal Sex (BCTS data)		
F	Female	83	0.39
M	Male	130	0.61
Missing		1	0.00

Table 5.2 continued - part 6.

Variable	Description	Number	Proportion
season	Season animal was slaughtered (abattoir data)		
Q1		83	0.39
Q2		61	0.29
Q3		41	0.19
Q4		29	0.14
Missing		0	0.00
breed	Animal Breed (abattoir data)		
Aberdeen Angus		24	0.11
Aberdeen Angus Cross		63	0.29
British Blue Cross		10	0.05
Charolais		1	0.00
Charolais Cross		18	0.08
Holstein Friesian		10	0.05
Limousin		4	0.02
Limousin Cross		40	0.19
Other		21	0.10
Simmental Cross		23	0.11
Missing		0	0.00
MS	Soil type (Macaulay Institute data)		
0	Urban area	5	0.02
1	Immature	9	0.04
3	Leached	122	0.57
4	Gleys	77	0.36
5	Organic soils	1	<0.01
Missing		0	0.00

Table 5.3: Variables included in model selection after univariable analysis.

Starting Model
fluke $\sim$ FarmerAgeR + PrevYearCows + ThisYearCalve + RiverDistance2 + BuyCattle + DeerPres + PrevYearCalv + Hay_Winter + Straw_Spring + AccessTempSource + FlDrugManag + sex + breed + age_days + season + year + PH_W_GMEAN_av + MS + MJJMeanTempMat + MJJMeanRainMat + (1|ProducerCode)

Table 5.4: Model selection using each of the 5 imputed datasets based on AIC values. Tables show which variable was removed at each step, the effect on the model AIC (Δ) and whether the variable was removed or kept in the model. Starting model is shown in 5.3.

Imputation 1	**AIC**	Δ	**Action**
Starting model	29322.3		
RiverDistance2	29320.3	$\Downarrow$	Removed
AccessTempSource	29318.3	$\Downarrow$	Removed
Hay_Winter	29316.3	$\Downarrow$	Removed
FarmerAgeR	29313.3	$\Downarrow$	Removed
PH_W_GMEAN_av	29311.7	$\Downarrow$	Removed
PrevYearCalv	29310.3	$\Downarrow$	Removed
PrevYearCows	29308.7	$\Downarrow$	Removed
breed	29306.7	$\Downarrow$	Removed
ThisYearCalve	29305.5	$\Downarrow$	Removed
MS	29305.5	$\Leftrightarrow$	Kept
FlDrugManag	29307.3	$\Uparrow$	Kept
sex	29307.3	$\Uparrow$	Kept
DeerPres	29309.7	$\Uparrow$	Kept

Imputation 2	**AIC**	Δ	**Action**
Starting Model	29315.2		
RiverDistance2	29313.2	$\Downarrow$	Removed
AccessTempSource	29311.3	$\Downarrow$	Removed
ThisYearCalve	29309.8	$\Downarrow$	Removed
PrevYearCows	29308.6	$\Downarrow$	Removed
PrevYearCalv	29308.4	$\Leftrightarrow$	Kept
PH_W_GMEAN_av	29308.5	$\Leftrightarrow$	Kept
FarmerAgeR	29307.7	$\Downarrow$	Removed
Hay_Winter	29307.6	$\Leftrightarrow$	Kept
DeerPres	29308.2	$\Uparrow$	Kept
sex	29308.4	$\Uparrow$	Kept
BuyCattle	29310.2	$\Uparrow$	Kept
MS	29307.9	$\Downarrow$	Removed
breed	29306.9	$\Downarrow$	Removed
Hay_Winter	29306.3	$\Leftrightarrow$	Kept
DeerPres	29306.5	$\Leftrightarrow$	Kept
PH_W_GMEAN_av	29307.2	$\Uparrow$	Kept

Imputation 3	**AIC**	Δ	**Action**
Starting Model	29316.3		
AccessTempSource	29314.3	$\Downarrow$	Removed
RiverDistance2	29312.4	$\Downarrow$	Removed
ThisYearCalve	29310.7	$\Downarrow$	Removed
FarmerAgeR	29307.1	$\Downarrow$	Removed
breed	29304.1	$\Downarrow$	Removed
MS	29302.4	$\Downarrow$	Removed
Hay_Winter	29300.5	$\Downarrow$	Removed
PrevYearCows	29299.0	$\Downarrow$	Removed
PrevYearCalv	29299.1	$\Leftrightarrow$	Kept
sex	29300.5	$\Uparrow$	Kept
FlDrugManag	29300.5	$\Leftrightarrow$	Kept
BuyCattle	29300.9	$\Leftrightarrow$	Kept

Imputation 4	**AIC**	Δ	**Action**
Starting Model	29316.7		
AccessTempSource	29316.7	$\Downarrow$	Removed
Hay_Winter	29312.9	$\Downarrow$	Removed
RiverDistance2	29311.1	$\Downarrow$	Removed
FarmerAgeR	29307.8	$\Downarrow$	Removed
PH_W_GMEAN_av	29306.4	$\Downarrow$	Removed
MS	29307.4	$\Uparrow$	Kept
PrevYearCalv	29307.2	$\Uparrow$	Kept
PrevYearCows	29307.3	$\Uparrow$	Kept
breed	29304.5	$\Downarrow$	Removed
ThisYearCalve	29305.0	$\Uparrow$	Kept
DeerPres	29305.4	$\Uparrow$	Kept

Imputation 5	**AIC**	Δ	**Action**
Starting Model	29316.4		
AccessTempSource	29314.5	$\Downarrow$	Removed
RiverDistance2	29312.7	$\Downarrow$	Removed
Hay_Winter	29311.2	$\Downarrow$	Removed
PrevYearCalv	29309.8	$\Downarrow$	Removed
PrevYearCows	29307.9	$\Downarrow$	Removed
breed	29306.0	$\Downarrow$	Removed
MS	29303.4	$\Downarrow$	Removed
FarmerAgeR	29300.6	$\Downarrow$	Removed
ThisYearCalve	29299.7	$\Downarrow$	Removed
DeerPres	29299.9	$\Leftrightarrow$	Kept
sex	29301.1	$\Uparrow$	Kept

Table 5.5: Variables remaining in each of the 5 imputed datasets based on model selection described in table 5.4.

IMPUTATION DATASET	Variables kept in each model	AIC
1	BuyCattle + DeerPres + Straw_Spring + season + year + MJJMeanTempMat + MJJMeanRainMat + age_days + FlDrugManag + sex	29305.5
2	BuyCattle + DeerPres + Straw_Spring + season + year + MJJMeanTempMat + MJJMeanRainMat + age_days + FlDrugManag + PrevYearCalv + PH_W_GMEAN_av + Hay_Winter	29306.9
3	BuyCattle + DeerPres + Straw_Spring + season + year + MJJMeanTempMat + MJJMeanRainMat + age_days + FlDrugManag + sex + PrevYearCalv + PH_W_GMEAN_av	29299
4	BuyCattle + DeerPres + Straw_Spring + season + year + MJJMeanTempMat + MJJMeanRainMat + age_days + FlDrugManag + sex + PrevYearCalv + ThisYearCalve + PrevYearCows + MS	29304.5
5	BuyCattle + DeerPres + Straw_Spring + season + year + MJJMeanTempMat + MJJMeanRainMat + age_days + FlDrugManag + sex + PH_W_GMEAN_av	29299.7

Table 5.6: Final model for the risk of liver rejection due to signs of *F. hepatica* infection in cattle slaughtered in Scotland.

Variable	Odds Ratio	Lower Level	Upper Level	p-value
Sex - Male	0.94	0.88	1.00	0.066
Soil pH	1.15	0.94	1.42	0.201
Buy Cattle - Yes	1.31	1.05	1.64	0.016
Deer Present - Yes	1.18	0.96	1.46	0.114
Straw supplemented in spring - Yes	0.79	0.64	0.97	0.022
Season Q2	0.94	0.87	1.01	0.078
Season Q3	0.73	0.67	0.80	<0.001
Season Q4	0.71	0.65	0.78	<0.001
Year - 2014	0.45	0.45	0.46	<0.001
Mean Temperature (May-Jun-Jul)	1.44	1.22	1.69	<0.001
Mean Rainfall (May-Jun-Jul)	1.02	1.01	1.03	0.001
Cattle age (days)	1.001	1.001	1.002	<0.001
Flukicides used on farm - Yes	1.30	1.01	1.68	0.046

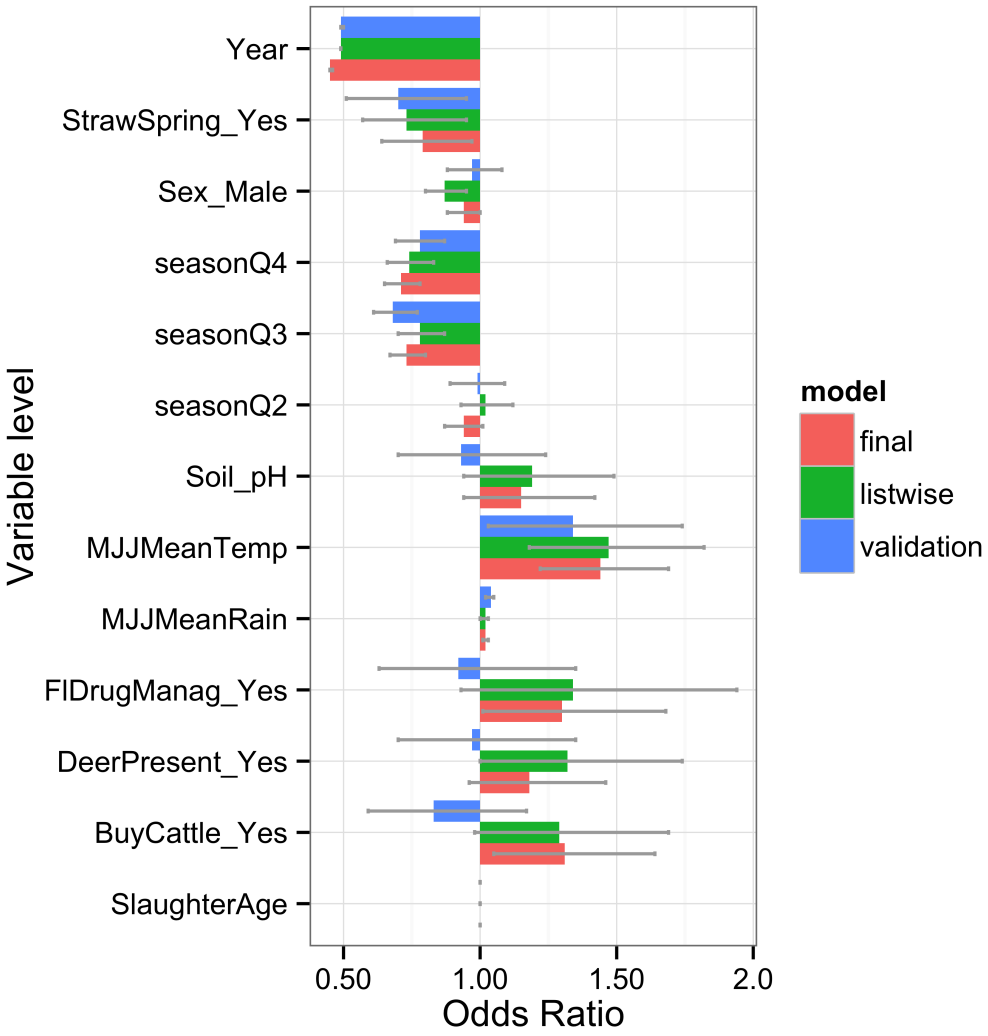


Figure 5.4: Comparison of final model results with validation and listwise models. Plot showing the results of running the same final model using the imputed training datasets (final model), the imputed validation datasets (validation model) and the dataset obtained by listwise deletion (listwise model). This shows that multiple imputation did not introduce bias to the results which are very comparable with listwise deletion, while it explains why the model has a moderate fit, as some estimates differ between the final model and the validation model.

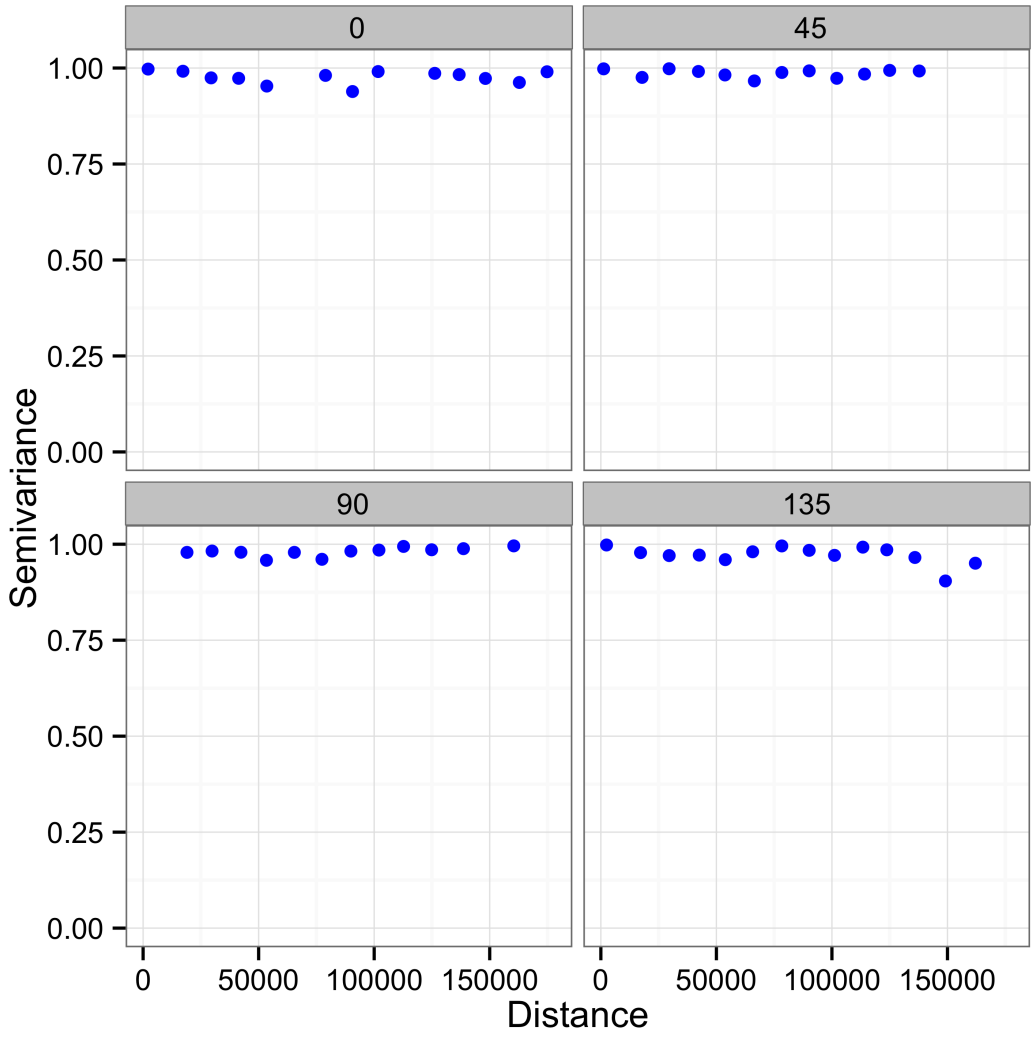


Figure 5.5: Variogram of model residuals. Residual semivariance was plotted in a northern, north-eastern, eastern, and south-eastern direction to check for spatial autocorrelation accounting for different directions. There is not obvious spatial autocorrelation.

5.5 Discussion

Several studies have predicted that the risk of fasciolosis will increase due to altered weather conditions related to global climate change (224; 43; 17; 49). Indeed in our study more than one in four livers were rejected due to signs of liver fluke in cattle consigned from Scottish holdings slaughtered between 2013 and 2014. The levels of infection were high in 2013 (30%) and did not reduce greatly in 2014 (20%). As discussed by Skuce et al. (167), this has been attributed to extreme weather events, more precisely, an extremely wet summer in 2012 followed by a very mild winter. This led to pastures being heavily contaminated with fluke and an increased risk of overwintering cysts, therefore prolonging the period animals were at risk of being infected (167). Cattle normally have one or two grazing seasons before they are slaughtered hence the consequences of these extreme weather conditions were seen in 2013, with a spillover in 2014.

These high proportions of rejected livers confirm the fact that there is a serious problem of fasciolosis in beef cattle in Scotland. The summary table of the questionnaire responses (Table 5.2) shows that while most of the farmers are aware of this problem, with many of them (75%) reporting the use of drugs to treat fluke, it is clear that improved guidance for sustainable management strategies is urgently required. For instance only 32% of producers reported that they use grazing management such as avoiding high risk areas to reduce the fluke burden on the farm. Additionally, only 22% of producers reported to adjust their treatment timings based on weather, only 9% said they used fluke forecasts to decide when to treat and only 18% monitor faecal egg counts.

In this study we built a multivariable mixed effects logistic regression model of the risk of liver fluke infection in beef cattle in an attempt to identify management strategies

that can help to reduce the risk of fasciolosis and therefore enable us to provide producers with better guidance on sustainable fluke management. The model included animal level data such as age, breed etc. that are routinely collected at the abattoir over a two year period in combination with management data, climatic and environmental data and investigated which of those variables can better predict the risk of liver fluke infection in each animal. This model has several strengths and limitations that are important to mention. The infection status of each animal was indicated by the result of liver inspection by the Meat Hygiene Service. This gave us the opportunity to have information about a very large number of animals covering a large geographical area. Nevertheless, one should remember that abattoir liver inspection has been estimated in chapter 3 of this thesis to have a sensitivity of 68% and a specificity of 88%, i.e. we expect a degree of misclassification in our outcome variable (225). Furthermore, as expected, the online administration of the questionnaire resulted in a significant proportion of missing data. This was dealt with using multiple imputation methodology in order to maintain all the producers completing the questionnaire, making the results more representative. It is important to ensure that this method did not introduce any bias, hence we fitted the final model on the same dataset using listwise deletion and showed that there were not important differences in the estimates of the two models.

The final model showed that animals which originated from farms with higher mean temperatures and rainfall had an increased risk of liver fluke infection. These factors appeared consistently important in the models from all five imputed datasets, which is in accordance with the work by McCann et al. (54) who showed that geographical and climate variables can explain 70-76% of variation in fluke infection in England and Wales. The final model only contains averages for May, June, July. As can be seen from the univariable analysis, this relationship remained the same for temperature and rainfall during other periods of the year, but as these variables were highly correlated

only one was maintained in the final model. The requirement for warm and moist environments for optimal transmission of *F. hepatica* in the UK has been recognized by Ollerenshaw more than half a century ago (15) and has been confirmed by various studies ever since. In the UK increasing temperatures and rainfall have been identified as risk factors for liver fluke infection in dairy herds in two recent studies (54; 197). Similarly, in neighbouring Ireland two recent studies by Selemetas et al. (226; 227) identified various measures of rainfall and temperature to be associated with higher risk of infection in Irish dairy herds. Furthermore, the increasing risk of fasciolosis with increasing soil pH can be supported by the fact that snail survival is favoured in alkaline water (228).

Our model also indicated that the risk of infection increased in cattle that originated from farms where cattle are bought in, farms where deer were present and farms that used flukicide to manage fluke problems on the farm. The risk of *F. hepatica* transmission due to animal movements is very intuitive and is supported by results from another abattoir based study in cattle in Denmark where unsurprisingly buying animals from positive herds was identified as a risk factor (229). In our study almost 70% of producers reported that they buy cattle in, while only half of those reported that they isolate bought in animals and 74% deworm new cattle. This is an area where educating producers on how to manage bought in cattle is crucial in order to decrease the risk of liver fluke and other infections. Furthermore, Rondelaud et al. (230) suggested that wild mammalian hosts such as rabbits and deer can act as reservoir of infection which can further complicate fasciolosis control (4). Indeed *F. hepatica* antibodies have been found in red, fallow and roe deer in previous studies in Spain (231; 232) although they have not been able to indicate an association between antibody levels in roe deer and antibody levels in cattle (232). This is an area where further research might be required to establish whether there is a real involvement of wildlife such as deer in the transmission of the parasite among domestic ruminants. The increased risk in cattle originating

from farms where flukicide treatments are in place is likely to be because farms who actually use anthelmintics, are more likely to be those who have a recognised liver fluke problem. Lastly, our model showed an increased risk with increasing age, which in the context of finishing beef cattle is possibly due to the fact that animals have a longer period in which they can get infected.

On the other hand our model indicated that male animals and animals from farms where straw is supplemented during spring appear to have a lower risk of fluke infection. Straw supplementation during spring might indicate that these animals are housed during high risk grazing periods, although we have no data to support this. Reduced risk of infection in male cattle has also been reported by Yildirim et al. (233) in a study in Turkey and reasons for this were thought to be different management of male and female animals. For instance, male animals are more likely to be housed than females.

According to our model the risk of fasciolosis in the UK depends mainly on climatic and environmental factors, the timing of slaughter which relates to the temporal risk of fasciolosis and animal characteristics. This is in disagreement with work by Bennema et al. (51) who found that when adjusting for management factors only rainfall remained important in their risk model of fasciolosis in dairy cattle in Belgium. In fact, they found that rainfall was negatively associated with the risk of infection. This emphasises the fact that the epidemiology of fluke might vary even between countries in temperate climate zones. Furthermore, it is possible that a study involving more detailed on farm recording of management strategies and potential environmental risks such as snail habitats, presence of water bodies etc might be more suitable for identifying farm specific management strategies which can help reduce the risk of fasciolosis in different farm settings (52; 61).

Overall, we have confirmed that climatic conditions play a very important role in the

risk of fasciolosis, along with cattle movements, and discussed a potential risk of the infection being transmitted by wildlife. As weather conditions remain favourable for fluke transmission, cattle movements increase and development of anthelmintic resistance continues, it is extremely important to increase producers' awareness of sustainable ways to control fluke. It is important that flukicides are used efficiently; using informed treatment times, and monitoring their efficiency either by using faecal egg counts and/or abattoir feedback. A greater emphasis needs to be given on pasture management strategies to avoid drug overuse, in order to reduce the development of anthelmintic resistance. Future work should focus on more detailed farm studies in order to identify finer scale indicators of fasciolosis risk, specific to different farm settings.

Chapter 6

Discussion

6.1 Introduction

At the beginning of this thesis we discussed the current situation regarding liver fluke in the UK, the challenges regarding liver fluke control and the prospect of using readily available abattoir data in order to fill identified research gaps towards improving liver fluke control strategies. The increase in prevalence and geographical spread of liver fluke in UK livestock during the last 15 years has been largely attributed to climate change, increased animal movements and emergence of triclabendazole resistance (31; 167). Based on predicted climate data it has been shown that the risk of liver fluke in parts of the UK will reach unprecedented levels and combined with predicted changes in the timing of disease outbreaks, will result in undermining currently practiced liver fluke control strategies (17). As challenges against the reduction of liver fluke prevalence in the UK continue to increase, improving our understanding of the parasite's changing epidemiology and our knowledge of the effect of infection on beef cattle production as well as about the diagnostic performance of available tests become of paramount importance for our efforts towards identifying sustainable control

measures for the reduction of the parasite's prevalence.

This thesis explored the value of slaughterhouse data in filling identified knowledge gaps using information collected at one of the largest cattle and sheep abattoirs in Scotland. The general aims of this thesis included; a) evaluation of available diagnostic tests and especially abattoir liver inspection, b) estimation of the difference in slaughter age between beef cattle infected with liver fluke vs. uninfected cattle, c) the investigation of the quantitative use diagnostic tests and d) the identification of risk factors associated with liver fluke infection.

6.2 Summary

In the introduction of this thesis (chapter 1), we introduced a simplified causal web to show the interrelationship between the parasite's life cycle, the mammalian host, management related factors and the environment (Figure 1.2). The dependence of the extra-mammalian stages of the parasite's development on both climatic parameters and the existence of an intermediate host (10; 3; 15) complicate the understanding of which factors are associated with an animal being infected with liver fluke. Management strategies are expected to vary both according to the anticipated risk of infection as well as the effect of infection on beef production. Understanding both the risk factors as well as the effect of infection on production is further compromised by uncertainty about the true infection status of an animal which depends on the accuracy of the diagnostic tests used. The thesis summary is shown in Figure 6.1, where we revisit the original diagram, adding new information that has arisen from each of the three data chapters of this thesis.

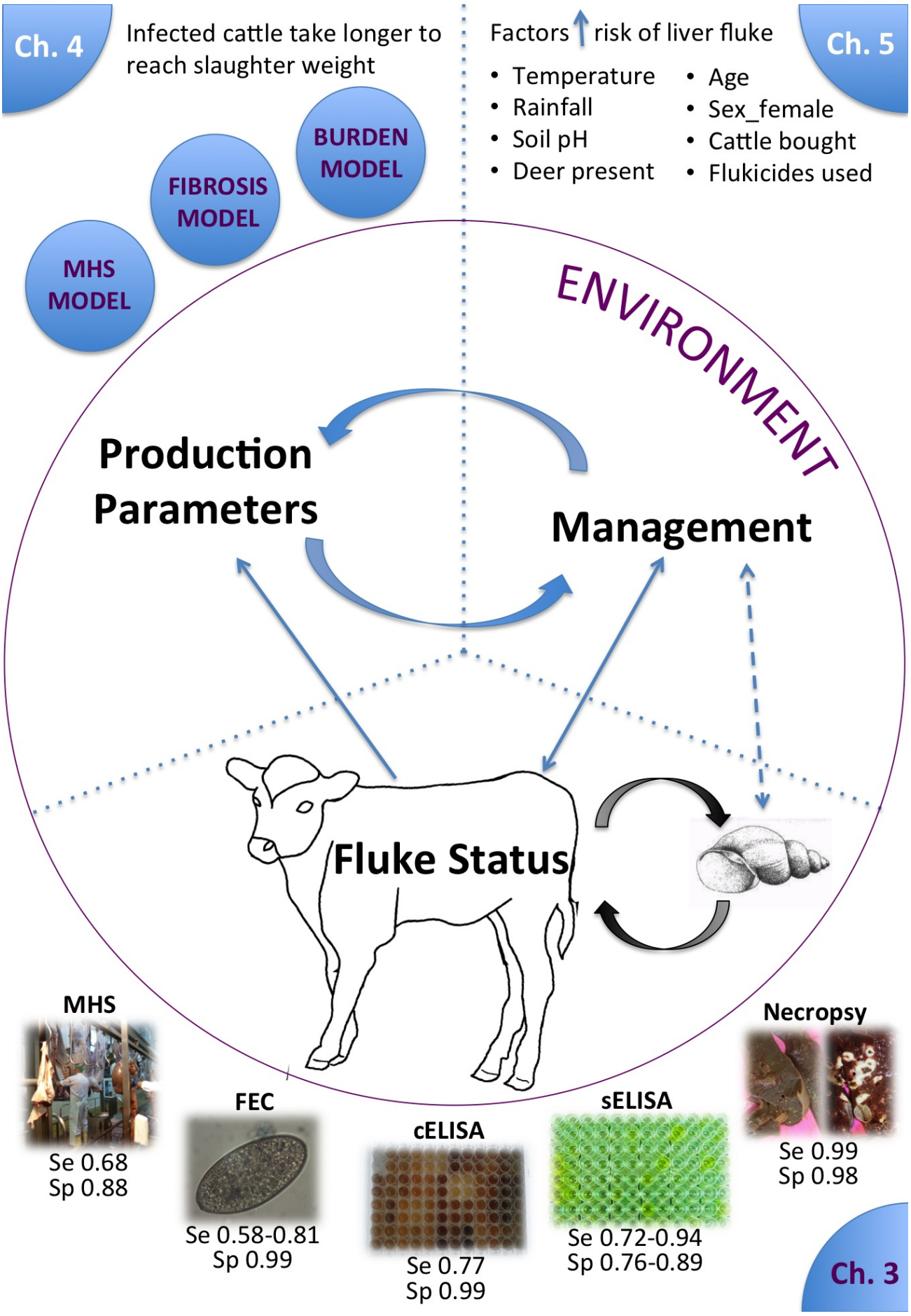


Figure 6.1: Thesis summary diagram.

In chapter 3 we used abattoir based methods to sample 619 cattle during three periods and analyse their liver fluke status with five different diagnostic tests. The results of the tests were then compared using a Bayesian no gold standard approach to estimate their diagnostic sensitivity and specificity. Detailed liver necropsy, including gall bladder egg counts, was used as a near perfect diagnostic test. This was undertaken to increase our understanding about the ability of the remaining test to distinguish between liver fluke infected and uninfected animals, which included faecal egg counting, a commercially available copro-antigen ELISA, an in-house serum excretory/secretory antibody ELISA and routine abattoir liver inspection.

The results provided novel information on the performance of these tests in a naturally infected cattle population at different times of the year. This is the first study to provide information on the accuracy of routine meat inspection for liver fluke in the UK. The inspection of livers for signs of *F. hepatica* infection is compulsory according to Regulation (EC) No 854/2004, hence data on the liver fluke rejection status of every single animal slaughtered at UK abattoirs is collected and can be potentially fed back to farmers or used to estimate the prevalence of fasciolosis. Accurate estimates of sensitivity and specificity are important as they can be used to adjust prevalences in these reports and improve the use of routine abattoir liver inspection as a tool for monitoring the epidemiology of *F. hepatica* and evaluating herd health planning. Faecal egg counting is possibly the most commonly used diagnostic test in practice and has been around for a long time. In our study we estimated the sensitivity of FEC during three different sampling periods. This was considered important as we have been able to show that while it is still a valuable tool in the diagnosis of current *F. hepatica* infections, FEC performs poorly during periods when infections in animals are recent. Very few studies have investigated the performance of the copro-antigen ELISA in cattle and we have been able to show that its sensitivity remains comparable throughout the year and have provided evidence to suggest that there is no cross-reaction with the increasingly

prevalent rumen fluke parasite. The last test evaluated in this study was an in house ES antigen ELISA developed by the Liverpool School of Tropical Medicine. Our study has shown that its sensitivity and specificity estimates are lower in the field setting than previously reported.

The main aim of chapter 4 was to estimate the difference in slaughter age between cattle infected with *F. hepatica* and uninfected cattle. In order to do this we fitted three different linear regression models, each including a different measure of liver fluke infection, and accounting for other important factors such as weight, age, sex, breed and farm as a random effect. The first model, the MHS model, used routinely collected abattoir data on meat inspection and carcass characteristics and estimated that cattle with livers rejected due to signs of liver fluke had on average 10 (95% CI 9-12) days greater slaughter age compared to animals who did not have their livers rejected, assuming an average carcass weight of 345 kg.

Data on the burden or extent of liver fluke infection in combination with carcass information of naturally infected cattle populations are scarce (170). Information on burden and fibrosis score were recorded through liver necropsy of each liver collected during the sampling. Combining these data with carcass data routinely collected at the abattoir provided us with a rare opportunity to investigate the difference in slaughter weight in cattle with different levels of burden and fibrosis. Our fibrosis model estimated that the increase in age at slaughter was more severe for higher fibrosis scores. More, precisely, the increase in slaughter age was 34 (95% CI 11-57) days for fibrosis score of 1, 93 (95% CI 57-128) days for fibrosis score 2 and 78 (95% CI 30-125) days for fibrosis score 3.

Morbidity and production losses are not necessarily related with parasite burden (234), and this relationship is likely to be complicated by the fact that the effect of liver flukes at different levels of burden depends on other factors such as duration of infection,

feed quality and whether animals are housed or not (191). Despite that, a cut-off of > 30 liver flukes was earlier suggested as an economic threshold (191). This was later challenged suggesting that the threshold might be as low as > 10 flukes (76). Our burden model compared animals with 1-10 parasites and animals with more than 10 parasites with animals that had no fluke burden. We estimated that there was a 31 (95% CI 7-56) days increase in slaughter age for animals with 1 to 10 parasites and 77 (95% CI 32-124) days increase in animals with more than 10 parasites found in their livers. These results support the suggested cut-off as it estimates that animals with a parasite burden higher than 10 take considerably longer to reach slaughter weight compared to uninfected animals. In fact, it is likely that the economic threshold is even lower, as animals with a burden of 1-10 parasites still appear to reach slaughter weight much later than uninfected animals.

Fasciolosis in cattle manifests mainly as a chronic sub-clinical disease, making production loss caused by the parasite less apparent to farmers. It has been argued that knowledge of the level of infection and the production cost associated to that can be more useful than simply knowing whether the infection is present or absent when trying to convince farmers to implement control measures (92). Having estimated the delay in slaughter for different levels of burden and fibrosis, the second part of chapter 4 investigated the relationship of continuous measures of FEC, the copro-antigen ELISA and the serum antibody ELISA with parasite burden and fibrosis score in an attempt to evaluate the ability of these tests to quantify parasite and morbidity levels corresponding to production losses as shown above. Positive correlations were identified both graphically and using Spearman rank correlation coefficients, but test result values at each level of both burden and fibrosis were very variable. Nevertheless, results of ROC curves used to evaluate the ability of a serum antibody ELISA, a copro-antigen ELISA and FEC to quantify liver fluke infection suggest that the tests evaluated can be useful in distinguishing between animals with production limiting levels of fibrosis and fluke

burden and animals with low or no liver damage or liver fluke burden.

Chapter 5, described the proportion of livers rejected due to signs of liver fluke in cattle slaughtered at Scotbeef that were consigned from Scottish based producers and identified risk factors associated with these rejections. Besides animal level data from cattle slaughtered during 2013 and 2014, we also used data collected using a herd management questionnaire as well as climatic and environmental data. More than one in four livers were rejected due to signs of liver fluke in cattle consigned from Scottish holdings slaughtered during the two year period. More precisely, the proportion of livers rejected reached 0.30 in 2013 and 0.20 in 2014, showing that liver fluke is a very common problem in the UK beef farming industry, hence guidelines for sustainable liver fluke control are urgently required.

A multi-variable mixed effects logistic regression model was built to estimate the association between climate, environmental, management and animal specific factors and the risk of an animal being infected by *F. hepatica*, using meat inspection results to determine the infection status of animals. Multiple imputation methodology was employed to deal with missing data arising from skipped questions in the questionnaire. Results of the regression model confirmed the importance of temperature, rainfall and cattle movements in increasing the risk for fasciolosis, while it indicated that the presence of deer can increase the risk of infection and that female cattle have an increased risk of infection. The types of variables retained in our final model indicated that climatic factors could better explain the risk of liver fluke in an individual animal. Nevertheless, other researchers have suggested that the difference in prevalence between closely located regions with similar climatic characteristics is possibly explained by different management strategies employed (53; 54), as was shown in previous studies in dairy cattle in Belgium (51; 52) and Sweden (145). Future studies can be designed to collect information through detailed on farm recording of management strategies

and potential environmental risks such as snail habitats, presence of water bodies etc, in order to identify farm specific management strategies which can help reduce the risk of fasciolosis in different farm settings (52; 61).

6.3 Limitations

The present study has several limitations which will be discussed in this section. The use of abattoir data provided us with a very efficient way to carry out epidemiological studies as the data are routinely collected for other purposes, hence they are readily available. Nevertheless, one has to bear in mind that there are some important limitations which are mainly related to how well the abattoir data represents the population of interest. For instance, the age structure of animals slaughtered at Scotbeef abattoir is likely to be different to the age structure of the general UK cattle population (125). Similarly, the representation of geographical regions is likely to be biased as the number of animals from each region are more likely to be related to factors such as distance from the abattoir rather than beef cattle population of that region (128). Furthermore, as the results of chapter 3 show meat inspection is an imperfect test, and unless accurate estimates of its ability to detect current infection are available, results can only relate to prevalence estimates (125) as we have no way to know at which point in time an animal was infected.

During the abattoir based sampling we have used systematic sampling, collecting a sample from every 10th animal slaughtered throughout the day. Since this is not a random sample, we do not attempt to describe the prevalence of the animals sampled. Nevertheless we believe that this was the optimum strategy that was logistically possible in order to represent the animals and herds slaughtered throughout the day. Furthermore the sample size used was specifically chosen for the no gold standard com-

parison of the five diagnostic tests (chapter 3) and was not necessarily the optimum sample size for the fibrosis and burden models of chapter 4. This is reflected by the wide confidence intervals for the estimates of the difference in age at slaughter for each level of fibrosis and burden respectively.

In chapter 5 of this thesis we combined abattoir data with results from producer questionnaires in an attempt to identify risk factors for the risk of liver fluke infection. This gave us the opportunity to have information about the fluke status and the management of a great number of animals. Nevertheless, this also introduced some important limitations. Firstly, as we have already established, meat inspection is imperfect hence using it to determine the infection status of animals is expected to introduce a degree of misclassification in our outcome variable (225). Secondly, the fact that questionnaires were administered online resulted in many questions being skipped and the sample of producers who completed it not being randomly selected. In order to maintain all producers who completed the questionnaire in the analysis, to avoid introducing further bias, multiple imputation methodology was used to deal with the missing data arising from skipped questions. Furthermore we compared important producer characteristics such as geographical distribution, number of animals slaughtered and proportion of animals with livers rejected due to signs of liver fluke with the overall producer characteristics to ensure they were not greatly different.

6.4 Public engagement

Throughout these four years one of the main aims of this work was to engage with producers and the abattoir industry as much as possible. This included presenting the project, at its early stages, at several producer meetings around the country, where the audience was informed about the project and especially about the producers question-

naire and encouraged to complete it. Furthermore, the author has attended the annual Scotbeef's Winter Fair every year since 2013, where our work was presented by means of posters, slide presentations and other visual aids and producers were able to discuss their concerns as well as be updated on the progress of the project. The author has also organised meetings with the meat inspectors in order to describe the project's aims, to identify practical ways to carry out the abattoir based sampling and to inform them about the results of this work.

Engagement with producers and other important stakeholders throughout the PhD has been very important. Firstly, it provided us with an insight into the producers' perspective on the problems associated with fasciolosis in the UK and prompted us to direct our research towards tangible outputs that could have a real impact on the day to day fasciolosis management. Questions on how any individual farmer's liver fluke problem compares to that of others, or whether fluke was actually causing any effect on cattle were frequently asked and it was apparent that obtaining feedback on the meat inspection results was invaluable to producers. Moreover, the rapport built between the author and the stakeholders facilitated the implementation of the project and built communication channels through which project outputs could be disseminated at the end of the project to those who deal with fasciolosis in their working environment.

6.5 Further work

In chapter 4 we investigated the difference in age at slaughter in animals with different liver fibrosis scores and discussed how important it would be for the producer to be able to receive feedback not only on the presence or absence of infection, but actually on the extent of infection and how that relates to production loss. Eradicating liver fluke is not possible for reasons already discussed, but we have shown that production

loss also depends on the severity of infection. It is therefore extremely important to be able to evaluate management strategies both in terms of reduction of the proportion of animals affected as well as in terms of the severity of infection in each animal.

In order to further verify the fibrosis model results using a greater sample size and to see whether fibrosis scoring could be used within the time frame available for routine meat inspection, the scoring system will be incorporated in the offal rejections recording system at the abattoir. The meat inspectors have been presented with the results of our analysis and were provided with written descriptions as well as photographic examples for each fibrosis score. The current system allows them to record whether the liver was rejected due to signs of active fluke or signs of historic fluke. This will be replaced with fibrosis scores 1, 2 and 3, while animals with no signs of fluke will be recorded as before. As we have seen in chapter 3 it is unclear whether "active" or "historic" is a useful classification as many of the livers classified as historic were found to harbour at least one fluke. This is further supported by the fact that during discussions with the meat inspectors it became apparent that the definition of active and historic varied greatly between inspectors.

We hope that a standardised system such as the one we are now developing, i.e. a simple fibrosis scoring system with standardised descriptions and photographic examples for each score, that all inspectors have access to, will improve the value of abattoir feedback provided to the producers. In this way, producers will be able to evaluate their liver fluke control strategies both in terms of the levels of fluke prevalence and also in terms of the extent of infection. Validation of the current fibrosis model, using real time fibrosis scores, will inform us as to whether we can use these scores to provide producers with information regarding the potential loss associated with each score.

Furthermore, in order to share the results of this work we plan to prepare a liver fluke

information leaflet which will include an overview of the project, describe the main results and explain the new scoring system. In short, the leaflet will emphasise the importance and interpretation of abattoir liver fluke rejection feedback, highlighting the fact that, similar to other available diagnostic tests, it is not perfect. Additionally, it will present the results on the estimated delay in slaughter associated with liver fluke infection and provide useful guidelines on sustainable liver fluke control based on the results of chapter 5 as well as general knowledge. The leaflet will be sent to the producers who completed the questionnaire either by email or post according to their preference recorded on page 1 of the questionnaire (Appendix A) and will be given out during Scotbeef's next Winter Fair.

6.6 Conclusion

Overall, this project has used slaughterhouse data to fill important knowledge gaps regarding *F. hepatica* infection in cattle. Using samples from naturally infected cattle, it has provided valuable information on the accuracy of routine abattoir meat inspection, as well as other tests available for liver fluke diagnosis in cattle. It has also provided estimates of the effect of infection on the time cattle take to reach slaughter weight at different levels of infection and identified relevant risk factors related to the infection. Following up on the project's outputs, a trial has been put in place, to validate the new liver rejection recording system proposed, where a standardised fibrosis score will be reorded for each liver without changing the currently practiced liver inspection methodology. Knowledge of the effect of infection on slaughter age, as well as regional risk factors for *F. hepatica* infection, along with an improved use of abattoir inspection results in the evaluation of treatment strategies, can provide farmers and veterinarians with better incentives and tools to improve their herd health strategies and in the longer term help reduce the incidence of liver fluke in cattle.

Appendices

Appendix A

Producer Questionnaire

Scotbeef Cattle Producers liver fluke questionnaire

A. Project Introduction

Dear Scotbeef producer,

I am Stella Mazeri, a postgraduate student at the University of Edinburgh Veterinary School, working on Scotbeef's project "Improved use of abattoir information to aid the management of liver fluke in cattle and sheep". There has been a huge increase in liver fluke throughout the UK with significant financial consequences. However we lack key information with which to identify effective control strategies. As part of our project we have developed a questionnaire with the aim to identify solutions to the problem of liver fluke on your farms.

This questionnaire has been put together by our team of vets at the University of Edinburgh and piloted with cattle farmers to ensure that it is useful and meaningful. It should take about 20 minutes to complete. Information collected will be used under strict confidentiality and your anonymity will be maintained when the results are presented. Only summary data and statistics will be reported and individuals will not be identified to Scotbeef or other third parties. The aim of this questionnaire is to collect accurate information on the different management practices among Scotbeef cattle producers. This will be combined with abattoir data on liver condemnations along with climatic data in order to identify high and low risk practices associated with liver fluke infection. Precise and complete answers are essential to ensure high quality, meaningful results, applicable to both producers and researchers. Your involvement in this project is both indispensable and greatly appreciated.

A written report with results from this project will be distributed upon the completion of the project. If interested please indicate your preferred method for receiving this report below.

1. Which is your preferred method for receiving the report?

- ○ Use my postal address to send me a paper copy of the report
- ○ Use my email address below to email me an electronic copy of the report
- ○ I do not wish to receive a report

Email Address:

Scotbeef Cattle Producers liver fluke questionnaire

B. Farm Details

This section is about general farm details. This will enable us to match this farm with movement as well as climatic data which we hope to analyse together to provide more meaningful results.

2. Farm details

Farm name	
Scotbeef producer code	
County Parish Holding (C.P.H) number	
Farm post code	
County	

Scotbeef Cattle Producers liver fluke questionnaire

C. Respondent's details

This section is about information regarding the person completing the questionnaire.

3. What is/are your first name(s)?

4. What is your last name?

5. What is your role on the farm (please tick all appropriate answers)?

- [ ] Owner
- [ ] Manager
- [ ] Other

Other (please specify)

6. Which category below includes your age?

- ○ 17 or younger
- ○ 18-20
- ○ 21-29
- ○ 30-39
- ○ 40-49
- ○ 50-59
- ○ 60 or older

7. How long have you owned/managed/worked on this farm?

years

Scotbeef Cattle Producers liver fluke questionnaire

D. Types and numbers of animals kept

This section is about the different types of animals kept on the farm and the approximate numbers of each species kept.

8. Which type of cattle herd do you run? (Please tick the correct answers from the options below. You can tick yes more than once)

	Yes	No
Cattle breeder	○	○
Cattle finisher	○	○
Dairy	○	○

9. What are the approximate numbers of the following categories of stock kept on this farm? (please complete the following text boxes by stating the approximate number of cattle on farm)

Cows	
Bulls	
Steers (1-2 years)	
Heifers (1-2 years)	
Calves (up to 1 year)	

What is the number of stock kept on this farm by breed? (please complete the following questions by stating the approximate number of cattle on farm)

10. Cows

Lim X	
Char X	
Simm X	
Angus X	
Herford X	
Limousin	
Charolais	
Simmental	
Angus	
Hereford	
Dairy	
Other	

Scotbeef Cattle Producers liver fluke questionnaire

11. Steers

Lim X

Char X

Simm X

Angus X

Herford X

Limousin

Charolais

Simmental

Angus

Hereford

Dairy

Other

12. Heifers

Lim X

Char X

Simm X

Angus X

Herford X

Limousin

Charolais

Simmental

Angus

Hereford

Dairy

Other

Scotbeef Cattle Producers liver fluke questionnaire

13. Calves

Lim X	
Char X	
Simm X	
Angus X	
Herford X	
Limousin	
Charolais	
Simmental	
Angus	
Hereford	
Dairy	
Other	

Scotbeef Cattle Producers liver fluke questionnaire

E. Management of bought in animals

This section is about how you manage animals you buy in.

14. Do you buy cattle in? (if the answer is no, please move to Section F)

- ○ Yes
- ○ No

15. How often do you buy cattle in?

- ○ Annually
- ○ More often than once per year
- ○ Less often than once per year

16. In the last 12 months how many animals were bought at a market?

Cows	
Bulls	
Steers (1-2 years)	
Heifers (1-2 years)	
Calves (up to 1 year)	

17. In the last 12 months how many animals were bought at a private sale?

Cows	
Bulls	
Steers (1-2 years)	
Heifers (1-2 years)	
Calves (up to 1 year)	

18. Do you deworm (against fluke and/or roundworms) new cattle brought onto the farm?

- ○ Yes
- ○ No

19. Which products do you use for deworming of new cattle?

Product 1	
Product 2	
Product 3	
No product used	

20. Do you isolate bought in cattle? (if no go to Q22)

- ○ Yes
- ○ No
- ○ Not applicable

21. How long do you usually isolate them for?

days

Scotbeef Cattle Producers liver fluke questionnaire

F. Other animals

22. The following questions refer to the presence of other animals on the farm

	Yes	No
Do you or other people keep sheep on the farm?	○	○
Do you or other people keep goats on the farm?	○	○
Do you or other people keep ducks on the farm?	○	○
Do you or other people keep geese on the farm?	○	○
Are ducks present on the farm or adjacent fields?	○	○
Are geese present on the farm or adjacent fields?	○	○
Are deer present on the farm or adjacent fields?	○	○
Are rabbits present on the farm or adjacent fields?	○	○

Scotbeef Cattle Producers liver fluke questionnaire

G. Grazing

23. Where are the calves/steers/heifers typically kept, month by month, throughout the year? (please tick any of the boxes appropriate in each month)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Housed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Unimproved hill (no drainage, lime or fertilizer)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Improved hill	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Permanent upland/lowland pasture	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Arable grazing (ploughed grass/clover)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

What do you mean by other?

24. Where are the cows typically kept, month by month, throughout the year? (please tick appropriate boxes)

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Housed	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Unimproved hill (no drainage, lime or fertilizer)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Improved hill	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Permanent upland/lowland pasture	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Arable grazing (ploughed grass/clover)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

What do you mean by other?

25. Please state the total area (owned or rented) by type used for cattle - please use acres

Unimproved hill

Improved hill

Permanent pasture

Arable grazing

26. Please state whether pastures used for cattle grazing in 2013 were grazed by cattle or other stock in the previous year.

	Yes	No	Not applicable
Cows	○	○	○
Calves	○	○	○
Steers/Heifers	○	○	○
Ewes	○	○	○
Lambs	○	○	○
Goats	○	○	○
Other animals	○	○	○

Other (please specify)

27. In 2013 which of the following animals grazed on the pasture calves/steers/heifers grazed on?

	Yes	No	Not applicable
Sheep	○	○	○
Horses	○	○	○
Calves	○	○	○
Goats	○	○	○
Other animals	○	○	○

Other (please specify)

28. Which of the following grazing systems do you use? (You can reply yes to more than one questions)

	Yes	No	Not applicable
Each animal species graze on separate fields	○	○	○
Rotational grazing	○	○	○
Co-grazing with sheep	○	○	○
Co-grazing with other species	○	○	○

Other (please specify)

29. Do you usually give any of the following supplementary feeding to your calves/steers/heifers? (please tick all appropriate boxes)

	Spring	Summer	Autumn	Winter
Hay	☐	☐	☐	☐
Silage	☐	☐	☐	☐
Straw	☐	☐	☐	☐
Concentrates	☐	☐	☐	☐

30. Have any of the pastures used for making hay suffered from flooding?

- ○ Yes
- ○ No
- ○ Not applicable

31. Have any of the pastures used for making silage suffered from flooding?

- ○ Yes
- ○ No
- ○ Not applicable

Scotbeef Cattle Producers liver fluke questionnaire

H. Pasture management

32. The following questions refer to pasture management

	Yes	No	Not applicable
Is farmyard manure spread on pasture?	○	○	○
Do you have wetland, marshland, seasonally flooded land or boggy areas?	○	○	○
Do you fence off water bodies (lochs, ponds, temporary flooded areas etc)?	○	○	○
Do you drain temporary water bodies?	○	○	○
Do animals have access to open water from temporary sources?	○	○	○
Do animals have access to open water from permanent sources (e.g. lochs etc)?	○	○	○

33. What percentage of the area available for cattle grazing is wetland, marshland, seasonally flooded land or boggy?

%

34. Are fields mowed or topped shortly before/during grazing by calves/steers/heifers?

- ○ Yes more than 50% of the pasture grazed by calves/steers/heifers
- ○ Yes less than 50% of the pasture grazed by calves/steers/heifers
- ○ No

Scotbeef Cattle Producers liver fluke questionnaire

I. Production

35. What was your spring calving percentage in 2013? (this question refers to the percentage of calves born relative to the number of cows/heifers mated)

%

36. What was your autumn calving percentage in 2013? (this question refers to the percentage of calves born relative to the number of cows/heifers mated)

%

37. What was the percentage of cow and/or heifers returning to oestrus during the last calving season?

%

Scotbeef Cattle Producers liver fluke questionnaire

J. Liver fluke management

38. The following questions are about liver fluke management.

	Yes	No	Not applicable
Do you actively manage your cattle herd to control liver fluke?	○	○	○
Do you use grazing management (avoiding high risk areas and/or times) to reduce the liver fluke burden on the farm?	○	○	○
Do you use flukicide (anti-fluke) drugs?	○	○	○

39. When and with what do you treat your cattle, yearlings and calves against liver fluke? (please write the code corresponding to the appropriate product from the table below)

	Cows	Steers/Heifers	Calves
January			
February			
March			
April			
May			
June			
July			
August			
September			
October			
November			
December			

Code	Product name	Code	Product name
A1	Albenil low dose oral suspension	I1	Ivomec Super injection
A2	Albenil oral suspension	L1	Levafas Diamond oral suspension
A3	Albex oral administration	N1	Norofas pour on
A4	Animec super solution for injection	O1	Ovispec S&C oral suspension
B1	Bimectin plus solution for injection	T1	Tribex oral suspension
C1	Closamectin pour on	T2	Triclacert oral suspension
C2	Closamectin solution for injection	T3	Trodax
C3	Combinex	V1	Vibramec Super solution for injection
C4	Cydectin Triclamox pour on	Z1	Zanil
E1	Endofluke oral suspension	OT	Other
F1	Fasinex oral suspension		

40. How do you determine the dose of flukicide to use for calves/steers/heifers (please tick all that apply)?

- [ ] Estimate weight of each animal
- [ ] Weigh and dose to average weight of herd
- [ ] Weigh and dose to heaviest animal
- [ ] Weigh individual animals
- [ ] I do not treat for fluke

Other (please specify)

41. How do you decide the timing of treatment each year?

	Yes	No	Not applicable
The timing of treatment is fixed	○	○	○
The timing of treatment depends on the weather	○	○	○
The timing of treatment depends on fluke forecast	○	○	○

Other (please specify)

42. Do you treat sheep for fluke?

- ○ Yes
- ○ No
- ○ No sheep kept on farm

Scotbeef Cattle Producers liver fluke questionnaire

K. Liver fluke history

43. The following questions are about liver fluke history.

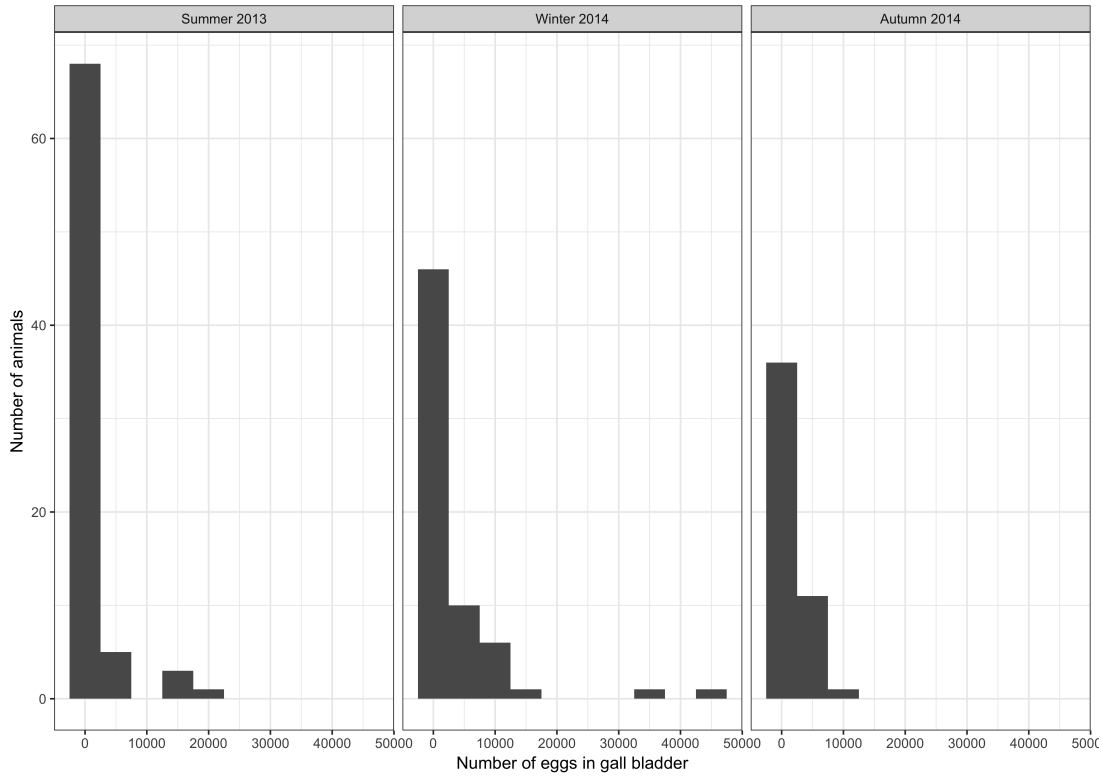
	Yes	No	Not applicable
Have you been aware of liver fluke problems in your cattle in the last year?	○	○	○
Have you been aware of liver fluke problems in your sheep in the last year?	○	○	○
Have you been aware of liver fluke problems in your cattle in the last five years?	○	○	○
Have you been aware of liver fluke problems in your sheep in the last five years?	○	○	○
Has liver fluke been diagnosed in your cattle by the vet in the last year?	○	○	○
Has liver fluke been diagnosed in your cattle by the vet in the last five years?	○	○	○
Do you monitor faecal egg counts?	○	○	○
Has liver fluke been reported in your cattle by the abattoir in the last year?	○	○	○
Has liver fluke been reported in your cattle by the abattoir in the last five years?	○	○	○

Appendix B

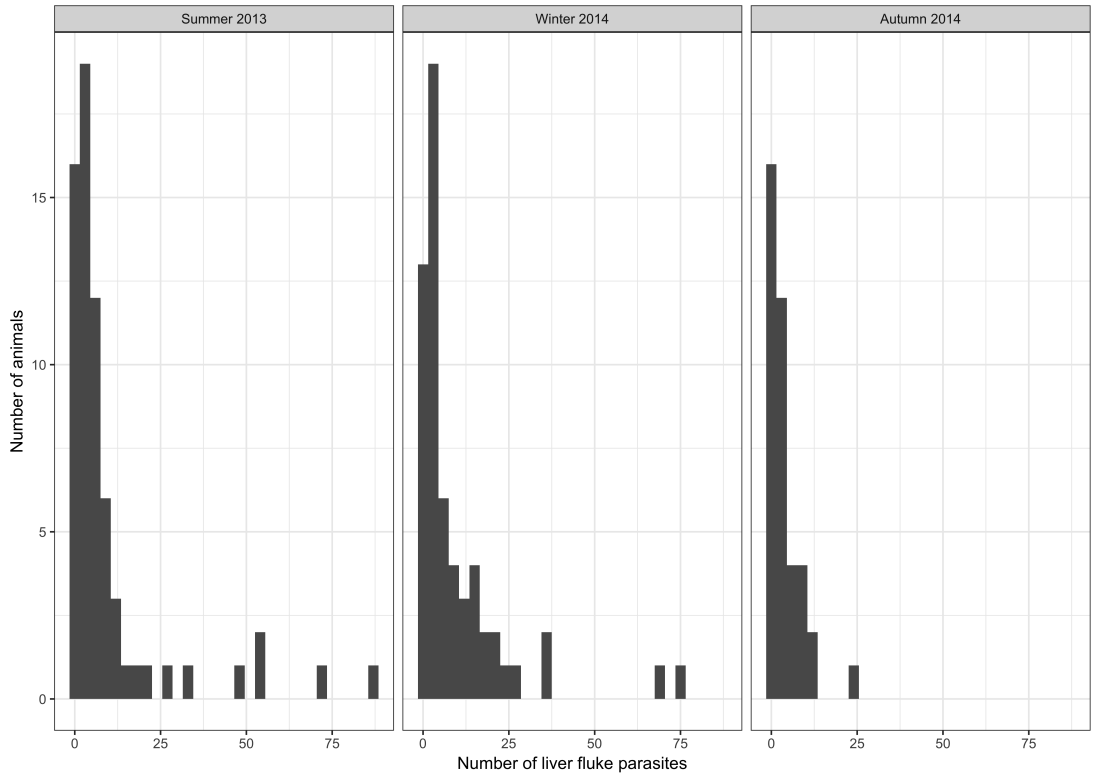
Additional Information for Chapter 3

Diagnostic test results - raw data

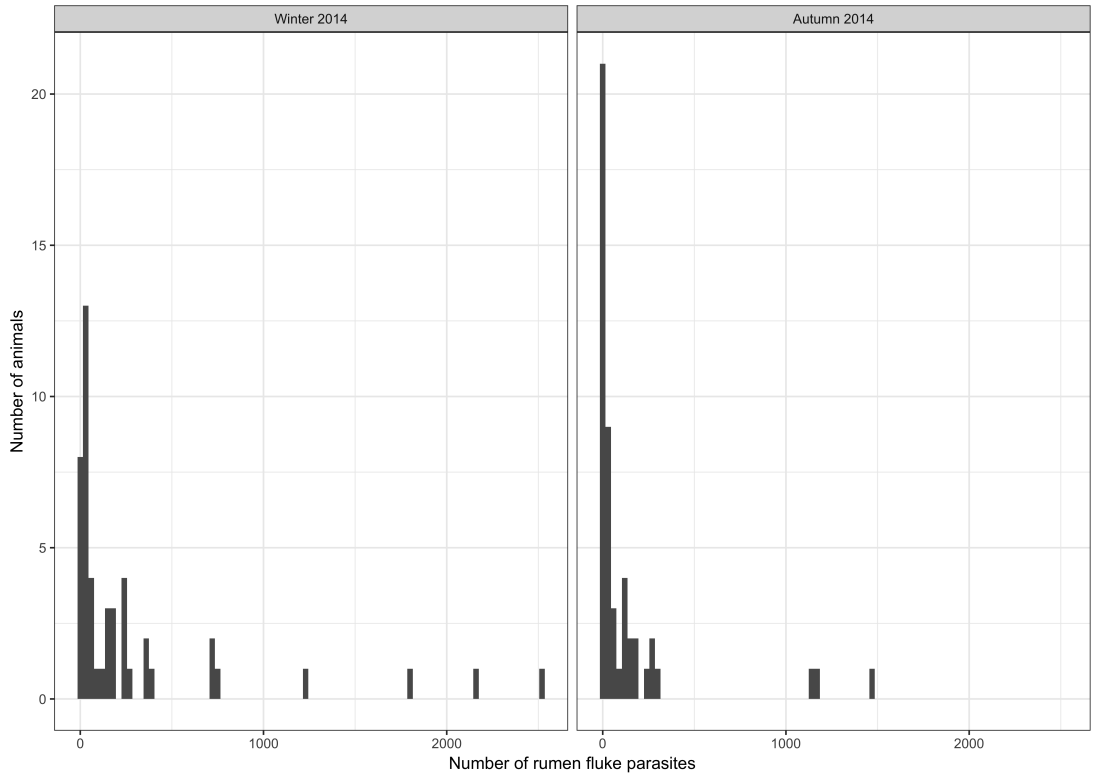
The following figures present the distributions of continuous diagnostic test results.



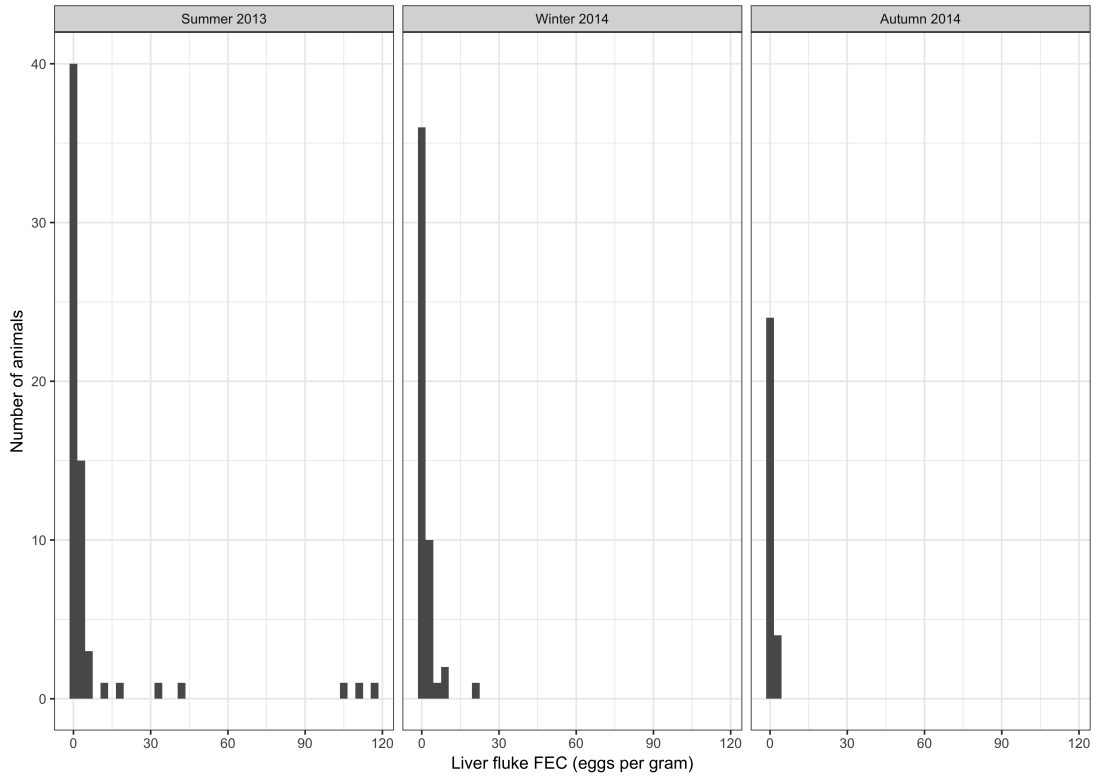
Distribution of liver fluke egg number found in gall bladder per sampling period. Figure includes animals with a liver fluke gall bladder egg number greater than 0.



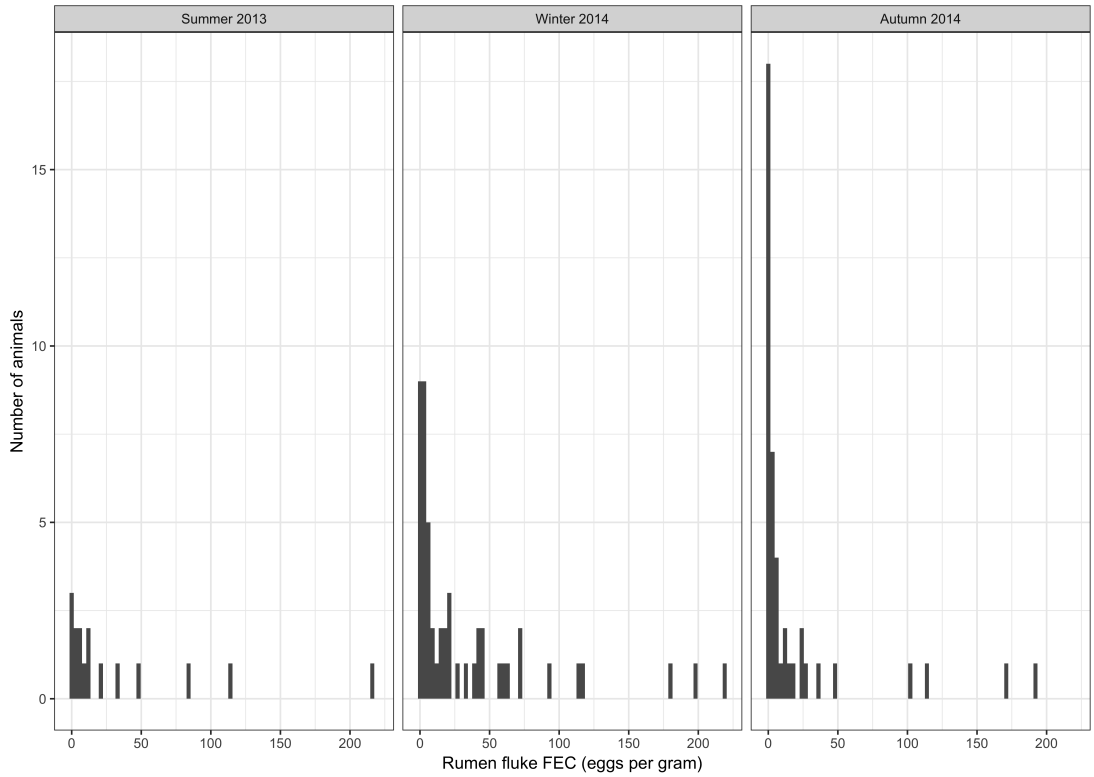
Distribution of liver fluke burden per sampling period. Figure includes animals with a liver fluke burden greater than 0.



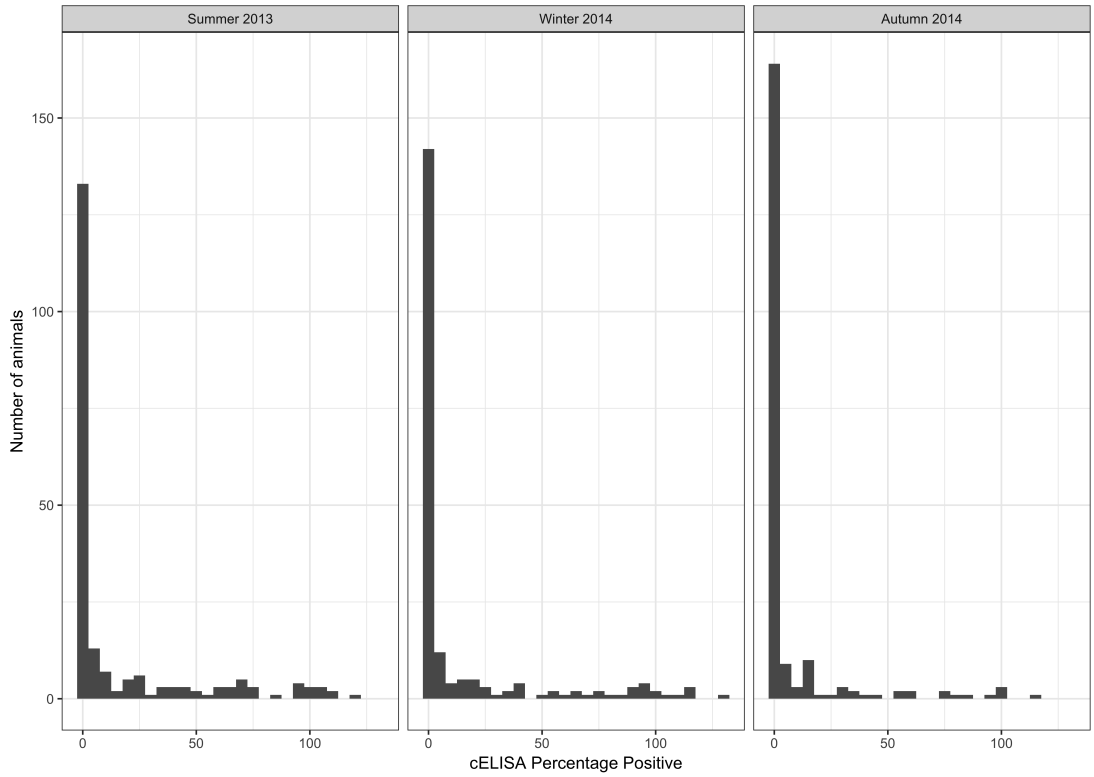
Distribution of rumen fluke burden per sampling period. Figure includes animals with a rumen fluke burden greater than 0.



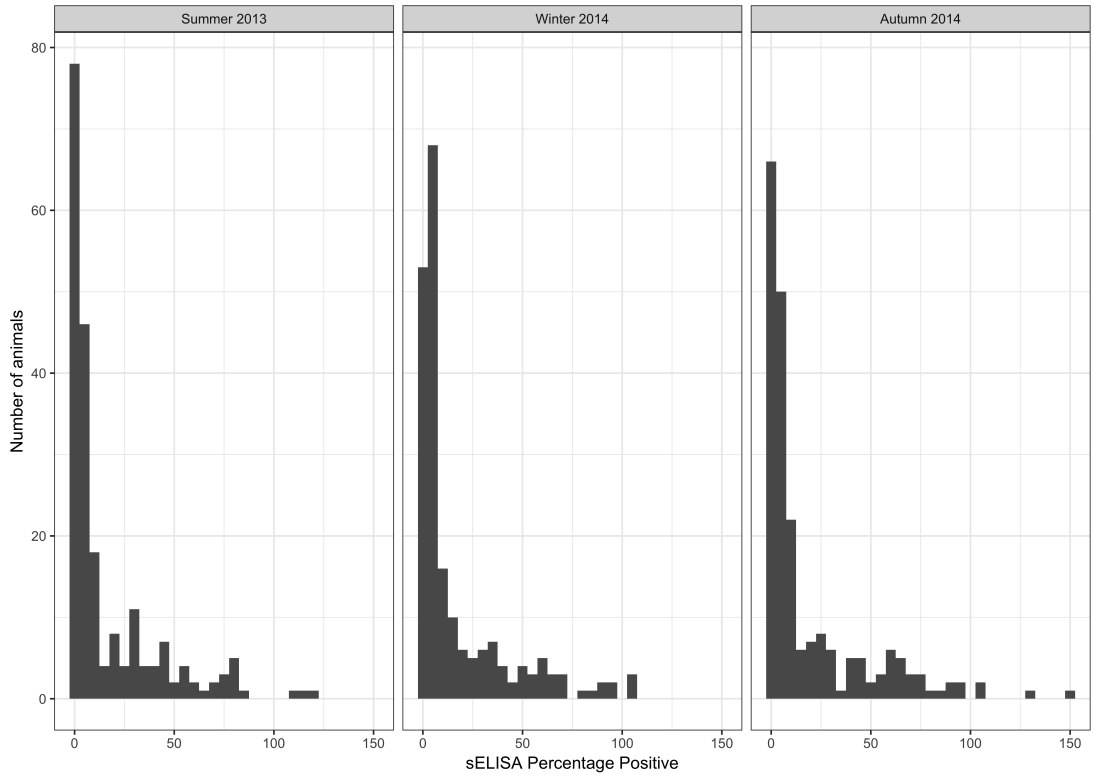
Distribution of liver fluke faecal egg count per sampling period. Figure includes animals with a faecal egg count greater than 0.



Distribution of rumen fluke faecal egg count per sampling period. Figure includes animals with a faecal egg count greater than 0.



Distribution of the copro-antigen ELISA percent positive per sampling period.



Distribution of the serum antibody ELISA percent positive per sampling period.

Model Specification

This script contains the code used for comparison of 5 diagnostic tests during 3 sampling periods. This model is an adaptation of the Hui & Walter (146) approach for the evaluation of diagnostic tests when a “gold standard" is not available.

```
model{

      yA[1:32] ~ dmulti(p[1:32], n1)


      p[32] <- piA  *  Se3 * Se4 * Se5 * Se1 * Se2                        + (1-piA)  *  (1-Sp3) * (1-Sp4) * (1-Sp5) * (1-Sp1) * (1-Sp2)
      p[31] <- piA  *  Se3 * Se4 * Se5 * (1-Se1) * Se2                    + (1-piA)  *  (1-Sp3) * (1-Sp4) * (1-Sp5) * Sp1 * (1-Sp2)
      p[30] <- piA  *  Se3 * Se4 * Se5 * Se1 * (1-Se2)                    + (1-piA)  *  (1-Sp3) * (1-Sp4) * (1-Sp5) * (1-Sp1) * Sp2
      p[29] <- piA  *  Se3 * Se4 * Se5 * (1-Se1) * (1-Se2)                + (1-piA)  *  (1-Sp3) * (1-Sp4) * (1-Sp5) * Sp1 * Sp2
      p[28] <- piA  *  (1-Se3) * Se4 * Se5 * Se1 * Se2                    + (1-piA)  *  Sp3 * (1-Sp4) * (1-Sp5) * (1-Sp1) * (1-Sp2)
      p[27] <- piA  *  (1-Se3) * Se4 * Se5 * (1-Se1) * Se2                + (1-piA)  *  Sp3 * (1-Sp4) * (1-Sp5) * Sp1 * (1-Sp2)
      p[26] <- piA  *  (1-Se3) * Se4 * Se5 * Se1 * (1-Se2)                + (1-piA)  *  Sp3 * (1-Sp4) * (1-Sp5) * (1-Sp1) * Sp2
      p[25] <- piA  *  (1-Se3) * Se4 * Se5 * (1-Se1) * (1-Se2)            + (1-piA)  *  Sp3 * (1-Sp4) * (1-Sp5) * Sp1 * Sp2
      p[24] <- piA  *  Se3 * (1-Se4) * Se5 * Se1 * Se2                    + (1-piA)  *  (1-Sp3) * Sp4 * (1-Sp5) * (1-Sp1) * (1-Sp2)
      p[23] <- piA  *  Se3 * (1-Se4) * Se5 * (1-Se1) * Se2                + (1-piA)  *  (1-Sp3) * Sp4 * (1-Sp5) * Sp1 * (1-Sp2)
      p[22] <- piA  *  Se3 * (1-Se4) * Se5 * Se1 * (1-Se2)                + (1-piA)  *  (1-Sp3) * Sp4 * (1-Sp5) * (1-Sp1) * Sp2
      p[21] <- piA  *  Se3 * (1-Se4) * Se5 * (1-Se1) * (1-Se2)            + (1-piA)  *  (1-Sp3) * Sp4 * (1-Sp5) * Sp1 * Sp2
      p[20] <- piA  *  (1-Se3) * (1-Se4) * Se5 * Se1 * Se2                + (1-piA)  *  Sp3 * Sp4 * (1-Sp5) * (1-Sp1) * (1-Sp2)
      p[19] <- piA  *  (1-Se3) * (1-Se4) * Se5 * (1-Se1) * Se2            + (1-piA)  *  Sp3 * Sp4 * (1-Sp5) * Sp1 * (1-Sp2)
      p[18] <- piA  *  (1-Se3) * (1-Se4) * Se5 * Se1 * (1-Se2)            + (1-piA)  *  Sp3 * Sp4 * (1-Sp5) * (1-Sp1) * Sp2
      p[17] <- piA  *  (1-Se3) * (1-Se4) * Se5 * (1-Se1) * (1-Se2)        + (1-piA)  *  Sp3 * Sp4 * (1-Sp5) * Sp1 * Sp2
      p[16] <- piA  *  Se3 * Se4 * (1-Se5) * Se1 * Se2                    + (1-piA)  *  (1-Sp3) * (1-Sp4) * Sp5 * (1-Sp1) * (1-Sp2)
      p[15] <- piA  *  Se3 * Se4 * (1-Se5) * (1-Se1) * Se2                + (1-piA)  *  (1-Sp3) * (1-Sp4) * Sp5 * Sp1 * (1-Sp2)
      p[14] <- piA  *  Se3 * Se4 * (1-Se5) * Se1 * (1-Se2)                + (1-piA)  *  (1-Sp3) * (1-Sp4) * Sp5 * (1-Sp1) * Sp2
      p[13] <- piA  *  Se3 * Se4 * (1-Se5) * (1-Se1) * (1-Se2)            + (1-piA)  *  (1-Sp3) * (1-Sp4) * Sp5 * Sp1 * Sp2
      p[12] <- piA  *  (1-Se3) * Se4 * (1-Se5) * Se1 * Se2                + (1-piA)  *  Sp3 * (1-Sp4) * Sp5 * (1-Sp1) * (1-Sp2)
      p[11] <- piA  *  (1-Se3) * Se4 * (1-Se5) * (1-Se1) * Se2            + (1-piA)  *  Sp3 * (1-Sp4) * Sp5 * Sp1 * (1-Sp2)
      p[10] <- piA  *  (1-Se3) * Se4 * (1-Se5) * Se1 * (1-Se2)            + (1-piA)  *  Sp3 * (1-Sp4) * Sp5 * (1-Sp1) * Sp2
      p[9]  <- piA  *  (1-Se3) * Se4 * (1-Se5) * (1-Se1) * (1-Se2)        + (1-piA)  *  Sp3 * (1-Sp4) * Sp5 * Sp1 * Sp2
      p[8]  <- piA  *  Se3 * (1-Se4) * (1-Se5) * Se1 * Se2                + (1-piA)  *  (1-Sp3) * Sp4 * Sp5 * (1-Sp1) * (1-Sp2)
      p[7]  <- piA  *  Se3 * (1-Se4) * (1-Se5) * (1-Se1) * Se2            + (1-piA)  *  (1-Sp3) * Sp4 * Sp5 * Sp1 * (1-Sp2)
      p[6]  <- piA  *  Se3 * (1-Se4) * (1-Se5) * Se1 * (1-Se2)            + (1-piA)  *  (1-Sp3) * Sp4 * Sp5 * (1-Sp1) * Sp2
      p[5]  <- piA  *  Se3 * (1-Se4) * (1-Se5) * (1-Se1) * (1-Se2)        + (1-piA)  *  (1-Sp3) * Sp4 * Sp5 * Sp1 * Sp2
      p[4]  <- piA  *  (1-Se3) * (1-Se4) * (1-Se5) * Se1 * Se2            + (1-piA)  *  Sp3 * Sp4 * Sp5 * (1-Sp1) * (1-Sp2)
      p[3]  <- piA  *  (1-Se3) * (1-Se4) * (1-Se5) * (1-Se1) * Se2        + (1-piA)  *  Sp3 * Sp4 * Sp5 * Sp1 * (1-Sp2)
      p[2]  <- piA  *  (1-Se3) * (1-Se4) * (1-Se5) * Se1 * (1-Se2)        + (1-piA)  *  Sp3 * Sp4 * Sp5 * (1-Sp1) * Sp2
      p[1]  <- piA  *  (1-Se3) * (1-Se4) * (1-Se5) * (1-Se1) * (1-Se2)    + (1-piA)  *  Sp3 * Sp4 * Sp5 * Sp1 * Sp2
```

```
yB[1:32] ~ dmulti(pB[1:32], n2)


pB[32] <- piB  *  Se3B * Se4B * Se5B * Se1 * Se2                    + (1-piB)  *  (1-Sp3) * (1-Sp4) * (1-Sp5B) * (1-Sp1) * (1-Sp2)
pB[31] <- piB  *  Se3B * Se4B * Se5B * (1-Se1) * Se2                + (1-piB)  *  (1-Sp3) * (1-Sp4) * (1-Sp5B) * Sp1 * (1-Sp2)
pB[30] <- piB  *  Se3B * Se4B * Se5B * Se1 * (1-Se2)                + (1-piB)  *  (1-Sp3) * (1-Sp4) * (1-Sp5B) * (1-Sp1) * Sp2
pB[29] <- piB  *  Se3B * Se4B * Se5B * (1-Se1) * (1-Se2)            + (1-piB)  *  (1-Sp3) * (1-Sp4) * (1-Sp5B) * Sp1 * Sp2
pB[28] <- piB  *  (1-Se3B) * Se4B * Se5B * Se1 * Se2                + (1-piB)  *  Sp3 * (1-Sp4) * (1-Sp5B) * (1-Sp1) * (1-Sp2)
pB[27] <- piB  *  (1-Se3B) * Se4B * Se5B * (1-Se1) * Se2            + (1-piB)  *  Sp3 * (1-Sp4) * (1-Sp5B) * Sp1 * (1-Sp2)
pB[26] <- piB  *  (1-Se3B) * Se4B * Se5B * Se1 * (1-Se2)            + (1-piB)  *  Sp3 * (1-Sp4) * (1-Sp5B) * (1-Sp1) * Sp2
pB[25] <- piB  *  (1-Se3B) * Se4B * Se5B * (1-Se1) * (1-Se2)        + (1-piB)  *  Sp3 * (1-Sp4) * (1-Sp5B) * Sp1 * Sp2
pB[24] <- piB  *  Se3B * (1-Se4B) * Se5B * Se1 * Se2                + (1-piB)  *  (1-Sp3) * Sp4 * (1-Sp5B) * (1-Sp1) * (1-Sp2)
pB[23] <- piB  *  Se3B * (1-Se4B) * Se5B * (1-Se1) * Se2            + (1-piB)  *  (1-Sp3) * Sp4 * (1-Sp5B) * Sp1 * (1-Sp2)
pB[22] <- piB  *  Se3B * (1-Se4B) * Se5B * Se1 * (1-Se2)            + (1-piB)  *  (1-Sp3) * Sp4 * (1-Sp5B) * (1-Sp1) * Sp2
pB[21] <- piB  *  Se3B * (1-Se4B) * Se5B * (1-Se1) * (1-Se2)        + (1-piB)  *  (1-Sp3) * Sp4 * (1-Sp5B) * Sp1 * Sp2
pB[20] <- piB  *  (1-Se3B) * (1-Se4B) * Se5B * Se1 * Se2            + (1-piB)  *  Sp3 * Sp4 * (1-Sp5B) * (1-Sp1) * (1-Sp2)
pB[19] <- piB  *  (1-Se3B) * (1-Se4B) * Se5B * (1-Se1) * Se2        + (1-piB)  *  Sp3 * Sp4 * (1-Sp5B) * Sp1 * (1-Sp2)
pB[18] <- piB  *  (1-Se3B) * (1-Se4B) * Se5B * Se1 * (1-Se2)        + (1-piB)  *  Sp3 * Sp4 * (1-Sp5B) * (1-Sp1) * Sp2
pB[17] <- piB  *  (1-Se3B) * (1-Se4B) * Se5B * (1-Se1) * (1-Se2)    + (1-piB)  *  Sp3 * Sp4 * (1-Sp5B) * Sp1 * Sp2
pB[16] <- piB  *  Se3B * Se4B * (1-Se5B) * Se1 * Se2                + (1-piB)  *  (1-Sp3) * (1-Sp4) * Sp5B * (1-Sp1) * (1-Sp2)
pB[15] <- piB  *  Se3B * Se4B * (1-Se5B) * (1-Se1) * Se2            + (1-piB)  *  (1-Sp3) * (1-Sp4) * Sp5B * Sp1 * (1-Sp2)
pB[14] <- piB  *  Se3B * Se4B * (1-Se5B) * Se1 * (1-Se2)            + (1-piB)  *  (1-Sp3) * (1-Sp4) * Sp5B * (1-Sp1) * Sp2
pB[13] <- piB  *  Se3B * Se4B * (1-Se5B) * (1-Se1) * (1-Se2)        + (1-piB)  *  (1-Sp3) * (1-Sp4) * Sp5B * Sp1 * Sp2
pB[12] <- piB  *  (1-Se3B) * Se4B * (1-Se5B) * Se1 * Se2            + (1-piB)  *  Sp3 * (1-Sp4) * Sp5B * (1-Sp1) * (1-Sp2)
pB[11] <- piB  *  (1-Se3B) * Se4B * (1-Se5B) * (1-Se1) * Se2        + (1-piB)  *  Sp3 * (1-Sp4) * Sp5B * Sp1 * (1-Sp2)
pB[10] <- piB  *  (1-Se3B) * Se4B * (1-Se5B) * Se1 * (1-Se2)        + (1-piB)  *  Sp3 * (1-Sp4) * Sp5B * (1-Sp1) * Sp2
pB[9]  <- piB  *  (1-Se3B) * Se4B * (1-Se5B) * (1-Se1) * (1-Se2)    + (1-piB)  *  Sp3 * (1-Sp4) * Sp5B * Sp1 * Sp2
pB[8]  <- piB  *  Se3B * (1-Se4B) * (1-Se5B) * Se1 * Se2            + (1-piB)  *  (1-Sp3) * Sp4 * Sp5B * (1-Sp1) * (1-Sp2)
pB[7]  <- piB  *  Se3B * (1-Se4B) * (1-Se5B) * (1-Se1) * Se2        + (1-piB)  *  (1-Sp3) * Sp4 * Sp5B * Sp1 * (1-Sp2)
pB[6]  <- piB  *  Se3B * (1-Se4B) * (1-Se5B) * Se1 * (1-Se2)        + (1-piB)  *  (1-Sp3) * Sp4 * Sp5B * (1-Sp1) * Sp2
pB[5]  <- piB  *  Se3B * (1-Se4B) * (1-Se5B) * (1-Se1) * (1-Se2)    + (1-piB)  *  (1-Sp3) * Sp4 * Sp5B * Sp1 * Sp2
pB[4]  <- piB  *  (1-Se3B) * (1-Se4B) * (1-Se5B) * Se1 * Se2        + (1-piB)  *  Sp3 * Sp4 * Sp5B * (1-Sp1) * (1-Sp2)
pB[3]  <- piB  *  (1-Se3B) * (1-Se4B) * (1-Se5B) * (1-Se1) * Se2    + (1-piB)  *  Sp3 * Sp4 * Sp5B * Sp1 * (1-Sp2)
pB[2]  <- piB  *  (1-Se3B) * (1-Se4B) * (1-Se5B) * Se1 * (1-Se2)    + (1-piB)  *  Sp3 * Sp4 * Sp5B * (1-Sp1) * Sp2
pB[1]  <- piB  *  (1-Se3B) * (1-Se4B) * (1-Se5B) * (1-Se1) * (1-Se2) + (1-piB)  *  Sp3 * Sp4 * Sp5B * Sp1 * Sp2
```

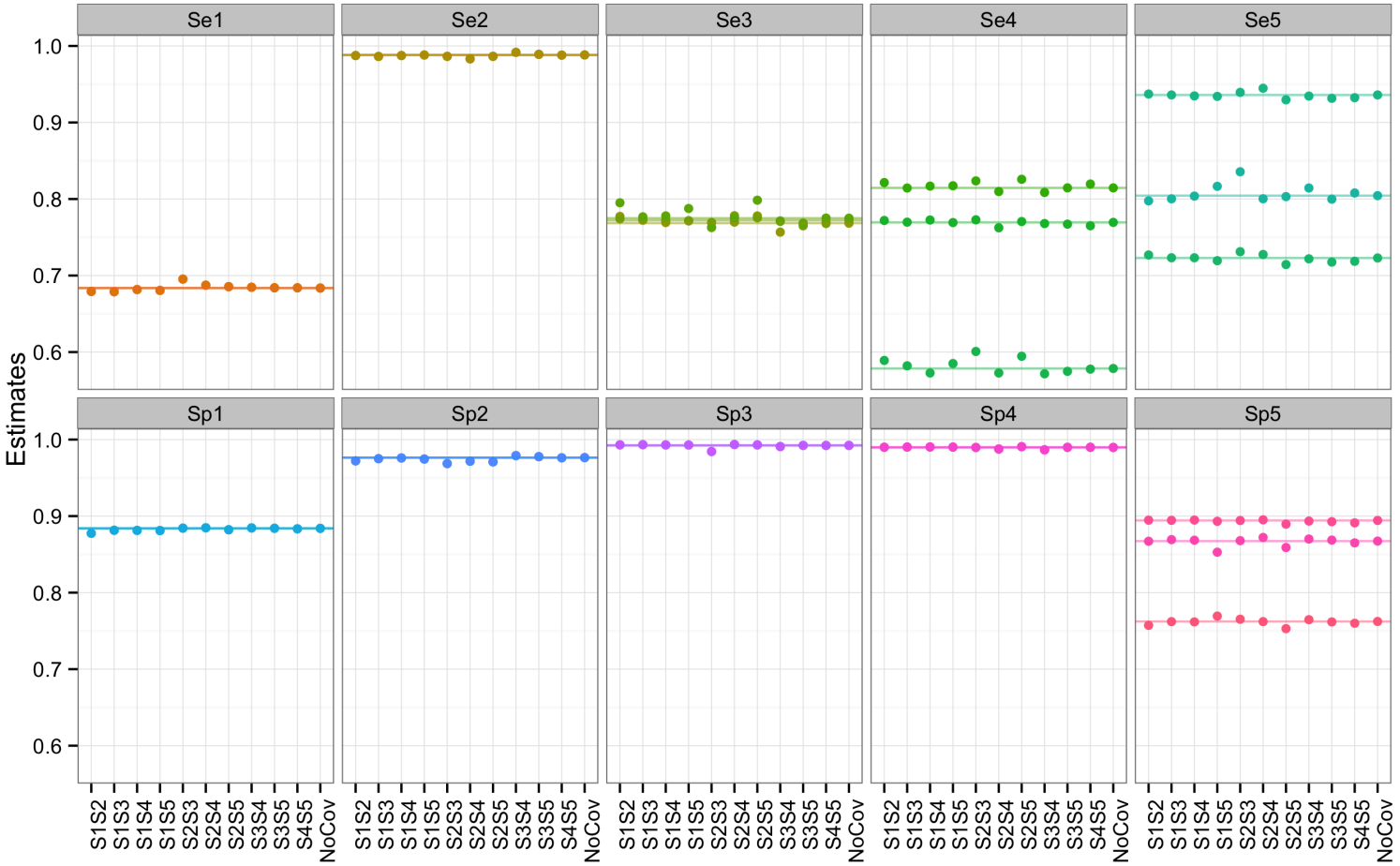
```
    yC[1:32] ~ dmulti(pC[1:32], n3)


    pC[32] <- piC  *  Se3C * Se4C * Se5C * Se1 * Se2                    + (1-piC)  *  (1-Sp3) * (1-Sp4) * (1-Sp5C) * (1-Sp1) * (1-Sp2)
    pC[31] <- piC  *  Se3C * Se4C * Se5C * (1-Se1) * Se2                + (1-piC)  *  (1-Sp3) * (1-Sp4) * (1-Sp5C) * Sp1 * (1-Sp2)
    pC[30] <- piC  *  Se3C * Se4C * Se5C * Se1 * (1-Se2)                + (1-piC)  *  (1-Sp3) * (1-Sp4) * (1-Sp5C) * (1-Sp1) * Sp2
    pC[29] <- piC  *  Se3C * Se4C * Se5C * (1-Se1) * (1-Se2)            + (1-piC)  *  (1-Sp3) * (1-Sp4) * (1-Sp5C) * Sp1 * Sp2
    pC[28] <- piC  *  (1-Se3C) * Se4C * Se5C * Se1 * Se2                + (1-piC)  *  Sp3 * (1-Sp4) * (1-Sp5C) * (1-Sp1) * (1-Sp2)
    pC[27] <- piC  *  (1-Se3C) * Se4C * Se5C * (1-Se1) * Se2            + (1-piC)  *  Sp3 * (1-Sp4) * (1-Sp5C) * Sp1 * (1-Sp2)
    pC[26] <- piC  *  (1-Se3C) * Se4C * Se5C * Se1 * (1-Se2)            + (1-piC)  *  Sp3 * (1-Sp4) * (1-Sp5C) * (1-Sp1) * Sp2
    pC[25] <- piC  *  (1-Se3C) * Se4C * Se5C * (1-Se1) * (1-Se2)        + (1-piC)  *  Sp3 * (1-Sp4) * (1-Sp5C) * Sp1 * Sp2
    pC[24] <- piC  *  Se3C * (1-Se4C) * Se5C * Se1 * Se2                + (1-piC)  *  (1-Sp3) * Sp4 * (1-Sp5C) * (1-Sp1) * (1-Sp2)
    pC[23] <- piC  *  Se3C * (1-Se4C) * Se5C * (1-Se1) * Se2            + (1-piC)  *  (1-Sp3) * Sp4 * (1-Sp5C) * Sp1 * (1-Sp2)
    pC[22] <- piC  *  Se3C * (1-Se4C) * Se5C * Se1 * (1-Se2)            + (1-piC)  *  (1-Sp3) * Sp4 * (1-Sp5C) * (1-Sp1) * Sp2
    pC[21] <- piC  *  Se3C * (1-Se4C) * Se5C * (1-Se1) * (1-Se2)        + (1-piC)  *  (1-Sp3) * Sp4 * (1-Sp5C) * Sp1 * Sp2
    pC[20] <- piC  *  (1-Se3C) * (1-Se4C) * Se5C * Se1 * Se2            + (1-piC)  *  Sp3 * Sp4 * (1-Sp5C) * (1-Sp1) * (1-Sp2)
    pC[19] <- piC  *  (1-Se3C) * (1-Se4C) * Se5C * (1-Se1) * Se2        + (1-piC)  *  Sp3 * Sp4 * (1-Sp5C) * Sp1 * (1-Sp2)
    pC[18] <- piC  *  (1-Se3C) * (1-Se4C) * Se5C * Se1 * (1-Se2)        + (1-piC)  *  Sp3 * Sp4 * (1-Sp5C) * (1-Sp1) * Sp2
    pC[17] <- piC  *  (1-Se3C) * (1-Se4C) * Se5C * (1-Se1) * (1-Se2)    + (1-piC)  *  Sp3 * Sp4 * (1-Sp5C) * Sp1 * Sp2
    pC[16] <- piC  *  Se3C * Se4C * (1-Se5C) * Se1 * Se2                + (1-piC)  *  (1-Sp3) * (1-Sp4) * Sp5C * (1-Sp1) * (1-Sp2)
    pC[15] <- piC  *  Se3C * Se4C * (1-Se5C) * (1-Se1) * Se2            + (1-piC)  *  (1-Sp3) * (1-Sp4) * Sp5C * Sp1 * (1-Sp2)
    pC[14] <- piC  *  Se3C * Se4C * (1-Se5C) * Se1 * (1-Se2)            + (1-piC)  *  (1-Sp3) * (1-Sp4) * Sp5C * (1-Sp1) * Sp2
    pC[13] <- piC  *  Se3C * Se4C * (1-Se5C) * (1-Se1) * (1-Se2)        + (1-piC)  *  (1-Sp3) * (1-Sp4) * Sp5C * Sp1 * Sp2
    pC[12] <- piC  *  (1-Se3C) * Se4C * (1-Se5C) * Se1 * Se2            + (1-piC)  *  Sp3 * (1-Sp4) * Sp5C * (1-Sp1) * (1-Sp2)
    pC[11] <- piC  *  (1-Se3C) * Se4C * (1-Se5C) * (1-Se1) * Se2        + (1-piC)  *  Sp3 * (1-Sp4) * Sp5C * Sp1 * (1-Sp2)
    pC[10] <- piC  *  (1-Se3C) * Se4C * (1-Se5C) * Se1 * (1-Se2)        + (1-piC)  *  Sp3 * (1-Sp4) * Sp5C * (1-Sp1) * Sp2
    pC[9]  <- piC  *  (1-Se3C) * Se4C * (1-Se5C) * (1-Se1) * (1-Se2)    + (1-piC)  *  Sp3 * (1-Sp4) * Sp5C * Sp1 * Sp2
    pC[8]  <- piC  *  Se3C * (1-Se4C) * (1-Se5C) * Se1 * Se2            + (1-piC)  *  (1-Sp3) * Sp4 * Sp5C * (1-Sp1) * (1-Sp2)
    pC[7]  <- piC  *  Se3C * (1-Se4C) * (1-Se5C) * (1-Se1) * Se2        + (1-piC)  *  (1-Sp3) * Sp4 * Sp5C * Sp1 * (1-Sp2)
    pC[6]  <- piC  *  Se3C * (1-Se4C) * (1-Se5C) * Se1 * (1-Se2)        + (1-piC)  *  (1-Sp3) * Sp4 * Sp5C * (1-Sp1) * Sp2
    pC[5]  <- piC  *  Se3C * (1-Se4C) * (1-Se5C) * (1-Se1) * (1-Se2)    + (1-piC)  *  (1-Sp3) * Sp4 * Sp5C * Sp1 * Sp2
    pC[4]  <- piC  *  (1-Se3C) * (1-Se4C) * (1-Se5C) * Se1 * Se2        + (1-piC)  *  Sp3 * Sp4 * Sp5C * (1-Sp1) * (1-Sp2)
    pC[3]  <- piC  *  (1-Se3C) * (1-Se4C) * (1-Se5C) * (1-Se1) * Se2    + (1-piC)  *  Sp3 * Sp4 * Sp5C * Sp1 * (1-Sp2)
    pC[2]  <- piC  *  (1-Se3C) * (1-Se4C) * (1-Se5C) * Se1 * (1-Se2)    + (1-piC)  *  Sp3 * Sp4 * Sp5C * (1-Sp1) * Sp2
    pC[1]  <- piC  *  (1-Se3C) * (1-Se4C) * (1-Se5C) * (1-Se1) * (1-Se2) + (1-piC)  *  Sp3 * Sp4 * Sp5C * Sp1 * Sp2

#Priors should be placed here
    }
```

Conditional Dependence

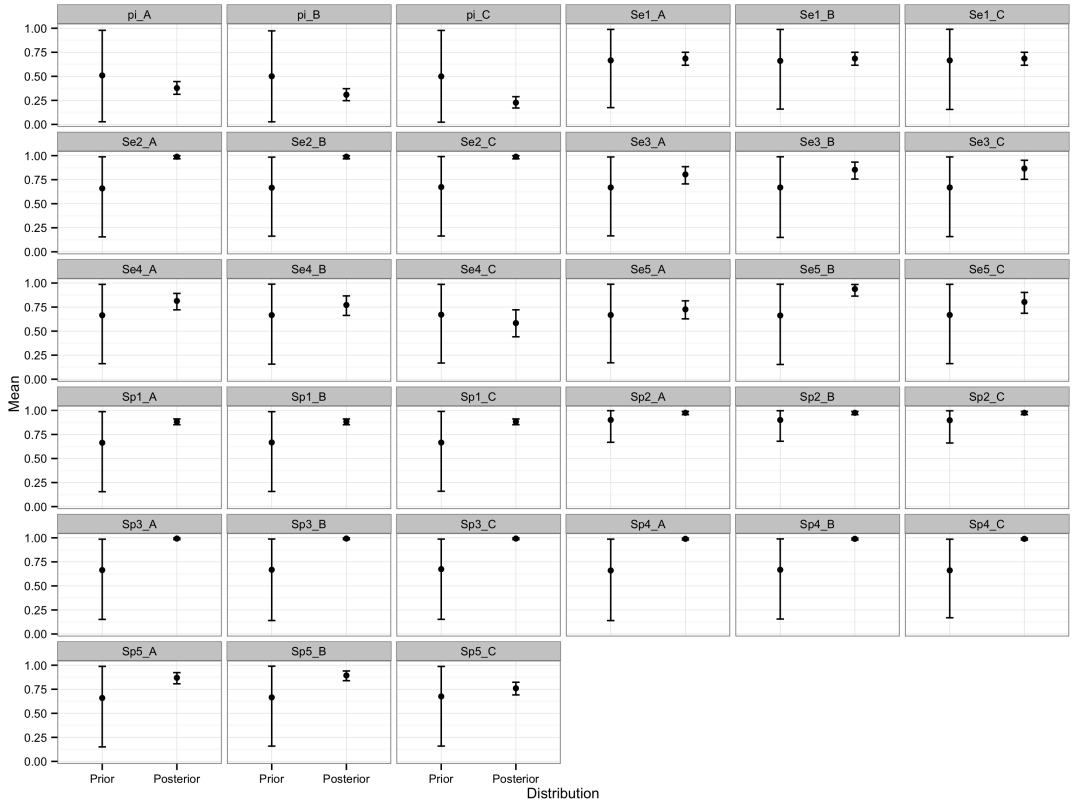
Figure shows the mean estimates of sensitivity and specificity of each test as estimated by the 10 different models accounting for covariance of one combination of two tests at a time. For example S1S2 is the model including covariance terms for tests 1 and 2 i.e. MHS liver inspection and liver necropsy and so on. The last estimate (NoCov) as well as the horizontal line on each plot shows the mean as estimated by the model with no covariance terms. Plots such as Se4 containing 3 lines show Se or Sp estimates that were allowed to vary between seasons. Based on this figure we concluded that even though estimates vary slightly above or below the lines, there are no major differences in estimates when accounting for covariance for the different combinations of tests and the model with no covariance terms. It was therefore justifiable to use a final model with no covariance terms.



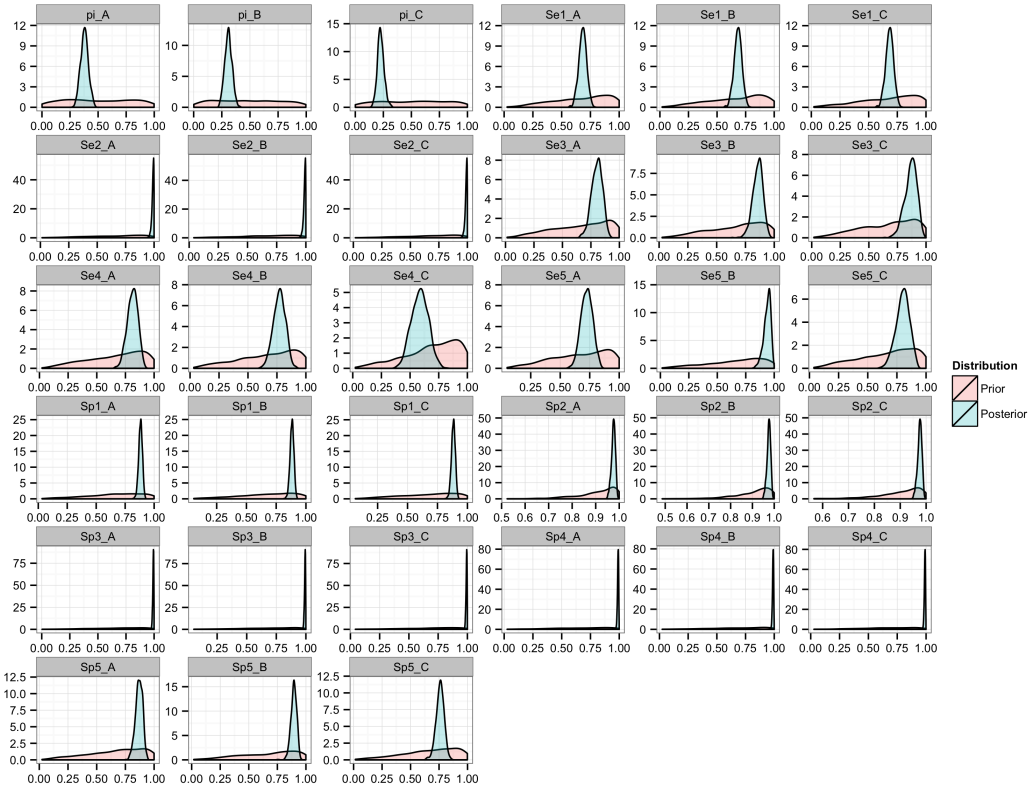
Mean estimates of sensitivity (Se) and specificity (Sp) for each test accounting for covariance for one combination of tests at a time. NoCov and straight lines show the estimate when no covariance terms are included (1 - MHS, 2 - Necropsy, 3 - cELISA, 4 - FEC, 5 - sELISA).

Effect of priors

A comparison between prior and posterior distributions of model parameters is shown in these two figures. The top figure shows the mean and 95% Bayesian credibility intervals of each model parameter. Bayesian credibility intervals of posterior distributions are much narrower than the priors showing that results are heavily informed by the data. As described in the methodology the only informative prior was the one for the specificity of the liver necropsy, Sp_2. This figure shows that even though the prior distribution is more informative the result is also informed by the data. Similarly the bottom figure shows the density plots of prior and posterior distributions and how prior distributions (except Sp_2) are vague and posterior distributions are highly data driven being much narrower than the prior distributions.



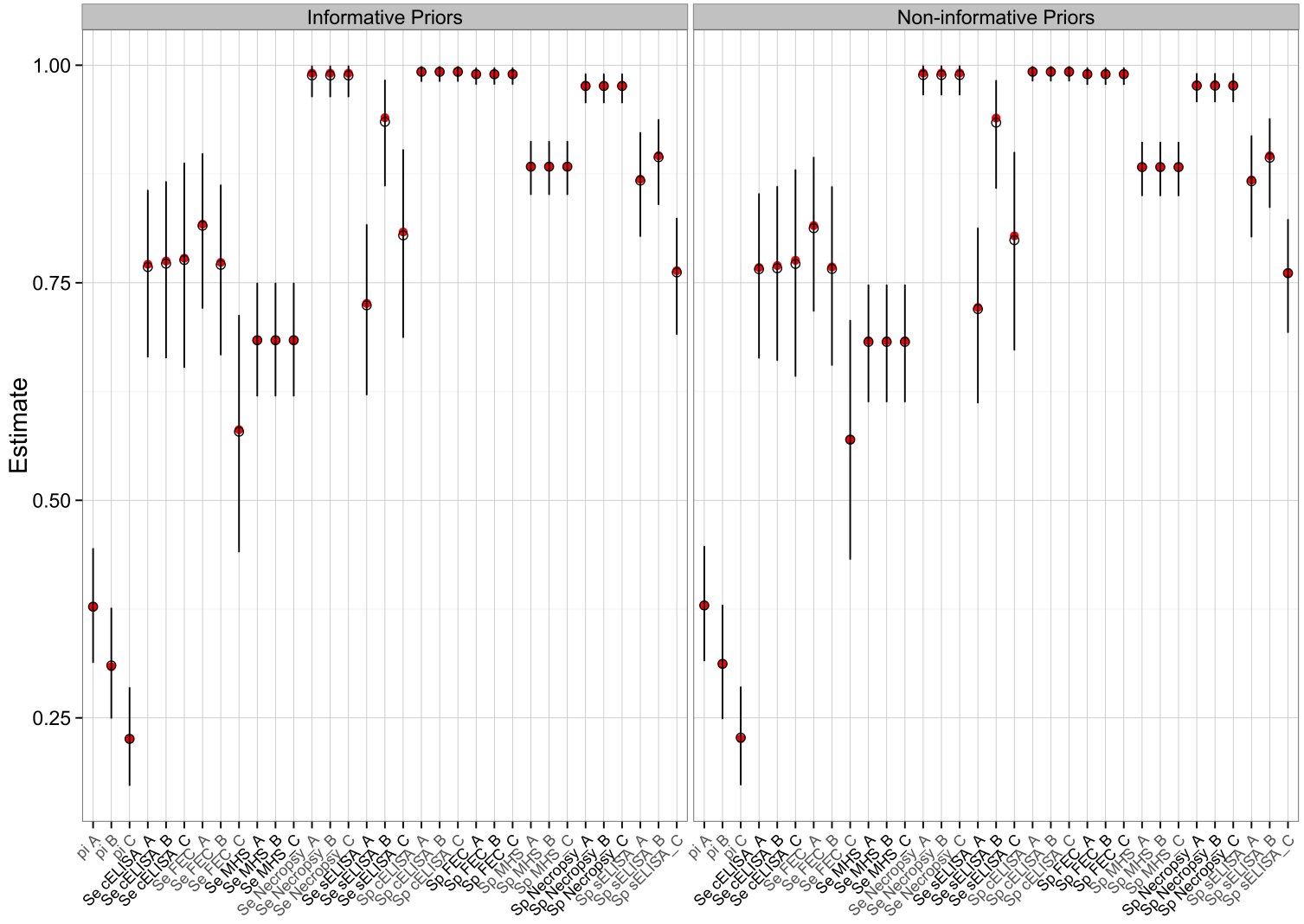
Comparison between prior and posterior distributions of model parameters by mean and 95% Bayesian credibility intervals. Key: pi - prevalence, 1 - MHS, 2 - Necropsy, 3 - cELISA, 4 - FEC, 5 - sELISA, A - summer 2013, B - winter 2014, C - autumn 2014.



Comparison between prior and posterior distributions of model parameters using density plots. Key: pi - prevalence, 1 - MHS, 2 - Necropsy, 3 - cELISA, 4 - FEC, 5 - sELISA, A - summer 2013, B - winter 2014, C - autumn 2014).

Comparison of results of original model and model using non-informative priors

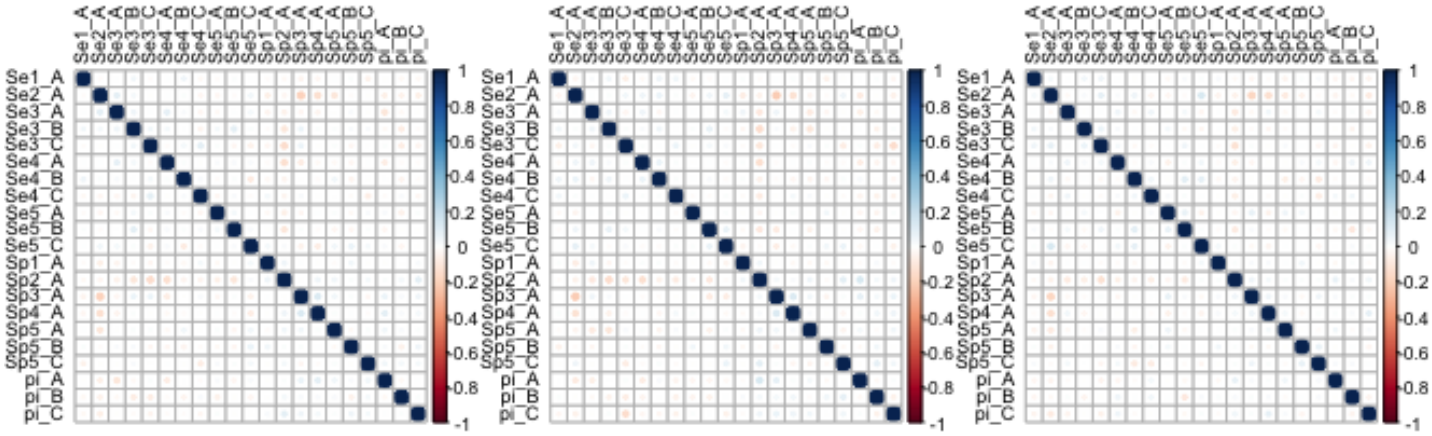
Figure shows the results of the original model and of a model using non-informative priors for comparison. The analysis was repeated using priors dbeta(1,1) for the Se and Sp of all tests to assess the effect of priors. There is no obvious alterations of results.



Comparison between results of the original model and of a model using non-informative priors.

Correlation between model parameters

Figure shows the cross correlations between the parameters included in the model in each of the 3 MCMC chains. There is no obvious strong correlation between any combination of parameters.



Correlation matrix of the variables included in the model for chains 1 to 3 (left to right). Key: Se - sensitivity, Sp - specificity, pi - prevalence, 1 - MHS, 2 - Necropsy, 3 - cELISA, 4 - FEC, 5 - sELISA, A - summer 2013, B - winter 2014, C - autumn 2014.

Data used in Bayesian no gold standard model

Table B.1: Data used in the Bayesian no gold standard model. For each period there were 32 possible combinations of test results and the number of animals for each combination is shown here. A negative test result is shown by 0 and a positive test result is shown by 1.

Combination	Test result (0=negative, 1=positive)					Number of animals		
	cELISA	FEC	sELISA	MHS	Necropsy	Period A	Period B	Period C
1	1	1	1	1	1	32	31	15
2	1	1	1	0	1	9	10	6
3	1	1	1	1	0	0	0	0
4	1	1	1	0	0	0	0	0
5	0	1	1	1	1	4	4	0
6	0	1	1	0	1	1	2	1
7	0	1	1	1	0	0	0	0
8	0	1	1	0	0	0	0	0
9	1	0	1	1	1	4	7	9
10	1	0	1	0	1	2	4	2
11	1	0	1	1	0	0	0	0
12	1	0	1	0	0	1	0	0
13	0	0	1	1	1	4	1	4
14	0	0	1	0	1	1	1	2
15	0	0	1	1	0	7	3	14
16	0	0	1	0	0	8	11	23
17	1	1	0	1	1	5	0	1
18	1	1	0	0	1	9	1	3
19	1	1	0	1	0	0	0	0
20	1	1	0	0	0	0	0	1
21	0	1	0	1	1	4	0	0
22	0	1	0	0	1	0	1	0
23	0	1	0	1	0	0	0	1
24	0	1	0	0	0	1	1	0
25	1	0	0	1	1	0	1	1
26	1	0	0	0	1	2	0	3
27	1	0	0	1	0	0	0	0
28	1	0	0	0	0	0	1	0
29	0	0	0	1	1	2	1	0
30	0	0	0	0	1	1	4	1
31	0	0	0	1	0	4	12	7
32	0	0	0	0	0	106	108	114

Appendix C

Additional Information for Chapter 4

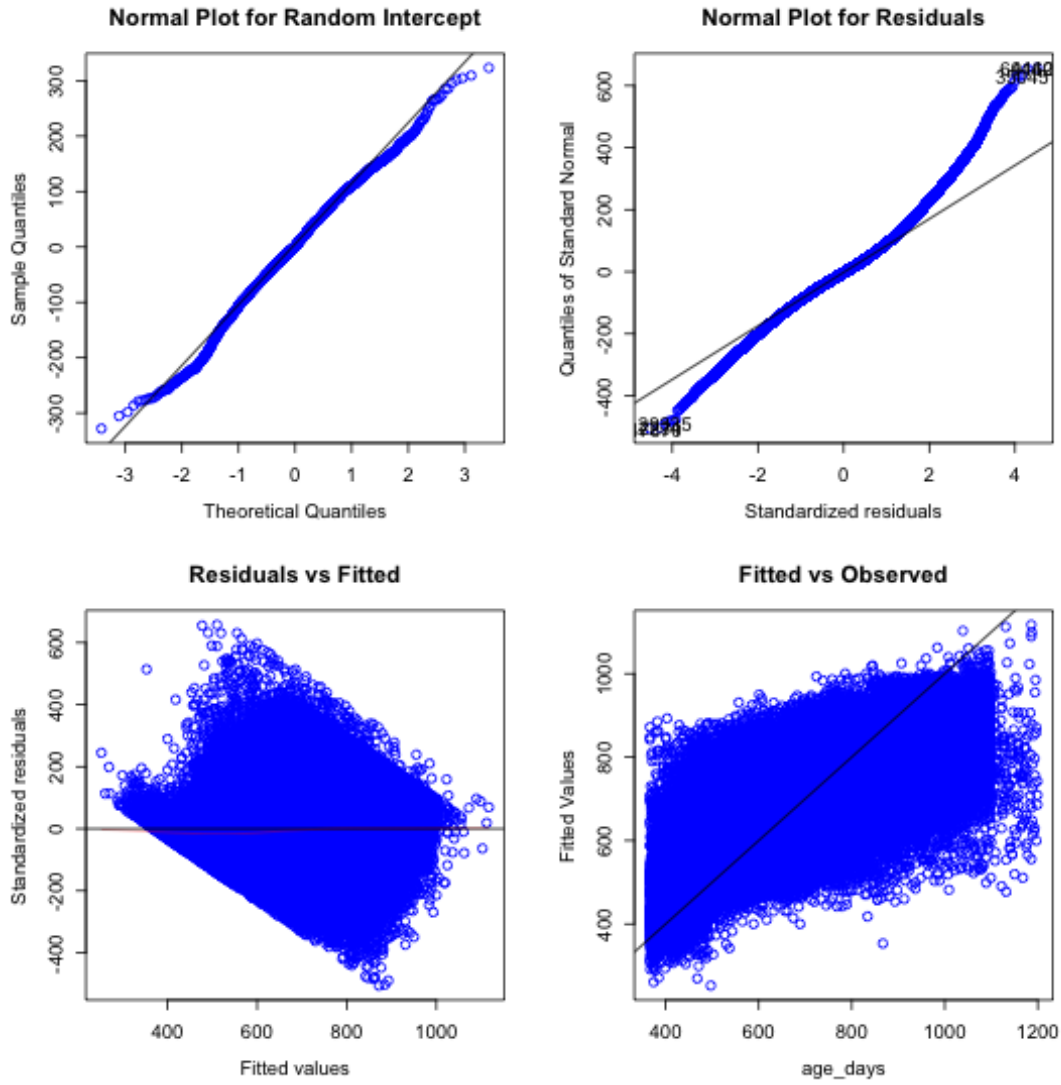


Figure C.1: MHS Model Diagnostic plots. Q-Q plot of random effect quantiles against theoretical quantiles to assess the normality of the random effect (top left). Q-Q plot of standard normal quantiles against standardized residuals (top right). Scatterplot of standardized residuals vs. fitted values to assess homoscedasticity of residuals (bottom left). Scatter plot of fitted values against observed values to assess predictive ability of the model (bottom right).

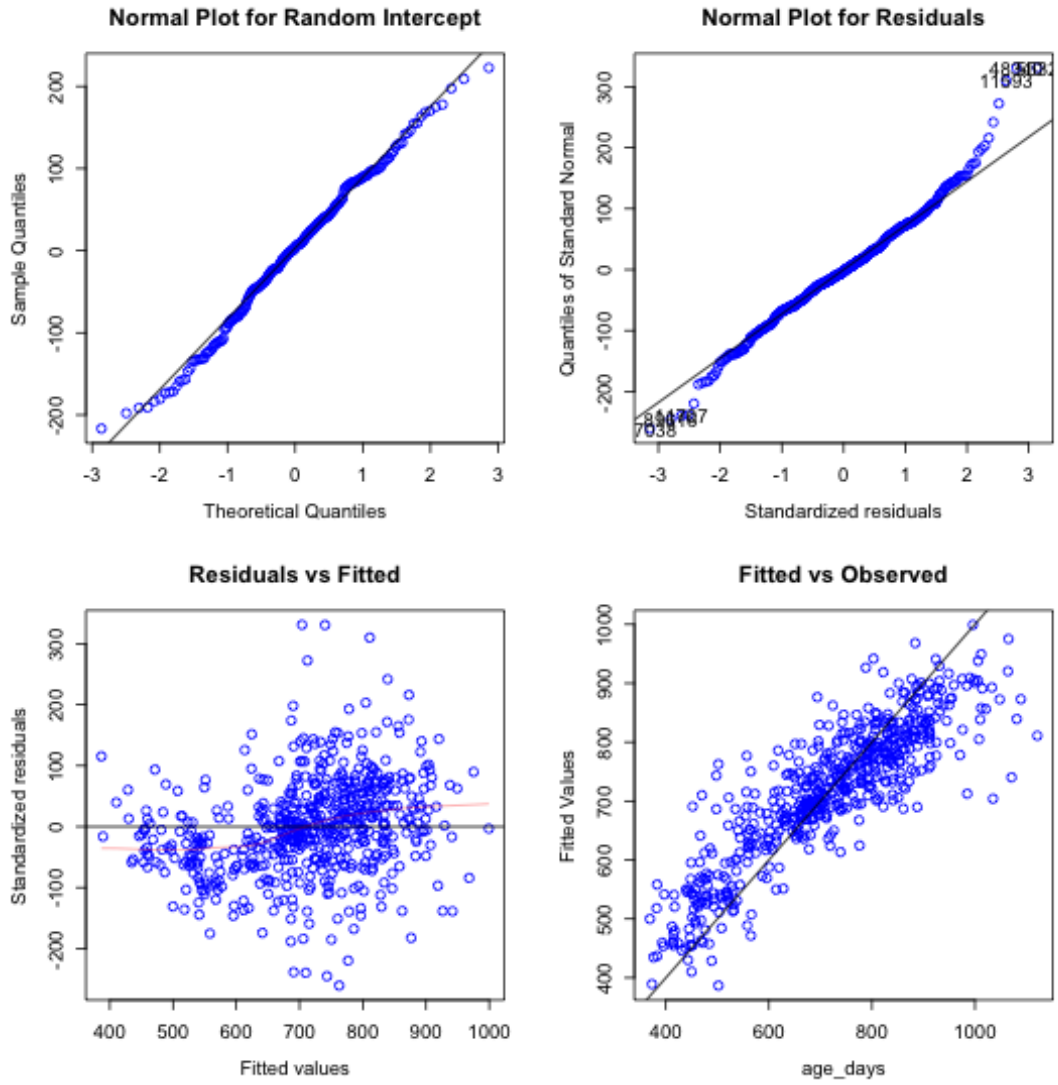


Figure C.2: Fibrosis Model Diagnostic plots. Q-Q plot of random effect quantiles against theoretical quantiles to assess the normality of the random effect (top left). Q-Q plot of standard normal quantiles against standardized residuals (top right). Scatter-plot of standardized residuals vs. fitted values to assess homoscedasticity of residuals (bottom left). Scatter plot of fitted values against observed values to assess predictive ability of the model (bottom right).

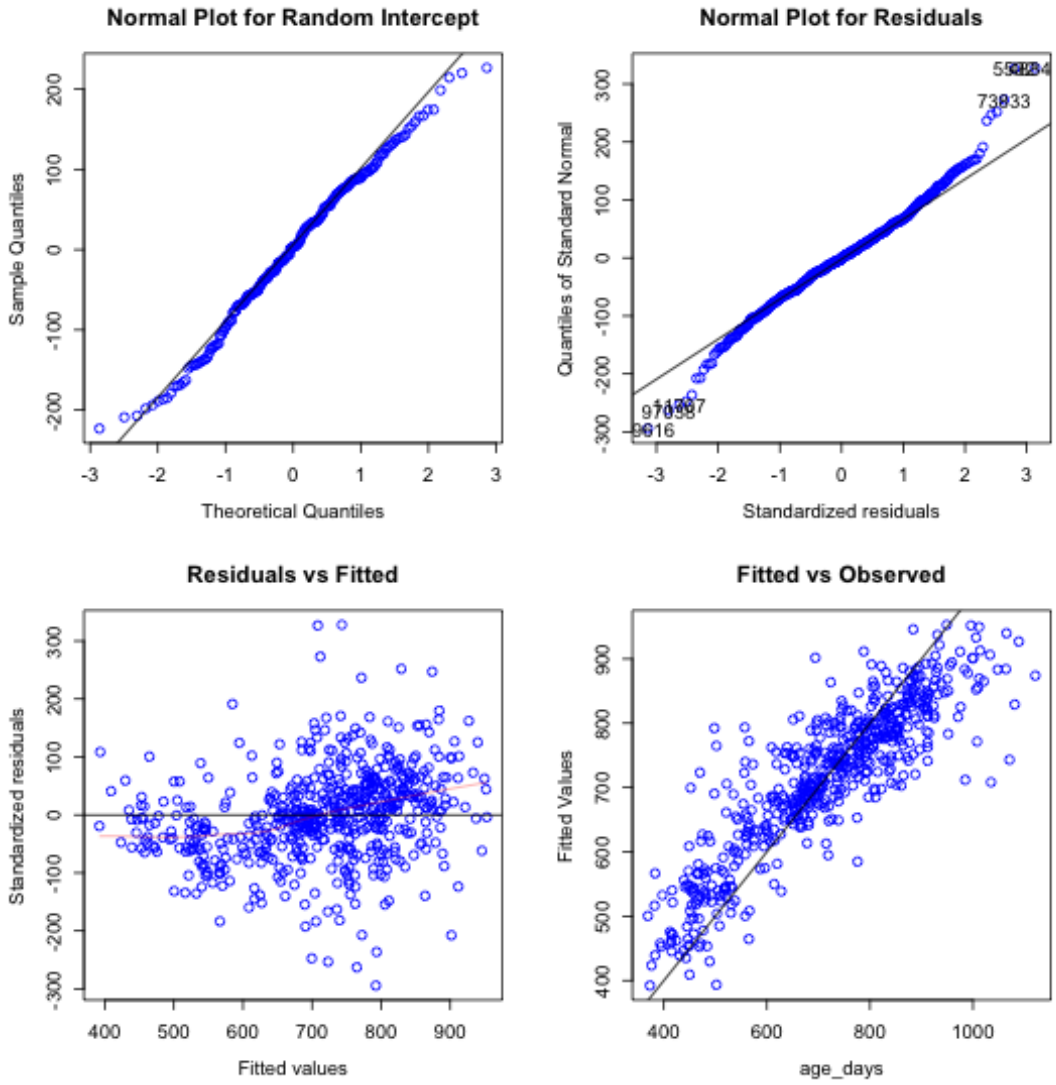


Figure C.3: Burden Model Diagnostic plots. Q-Q plot of random effect quantiles against theoretical quantiles to assess the normality of the random effect (top left). Q-Q plot of standard normal quantiles against standardized residuals (top right). Scatter-plot of standardized residuals vs. fitted values to assess homoscedasticity of residuals (bottom left). Scatter plot of fitted values against observed values to assess predictive ability of the model (bottom right).

Appendix D

Additional Information for Chapter 5

Table D.1: Results of univariable analysis for the risk of liver rejection due to signs of *F. hepatica* infection in cattle slaughtered in Scotland. The first level shown for each variable was used as the reference levels. Table also included cross tabulation of categorical variables against presence or absence of fluke.

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
FarmerAgeR						0.188
<30	531	1062				
30-49	5955	16361	0.90	0.49	1.66	0.740
>49	4690	12337	0.72	0.39	1.33	0.293
HerdTypeR						0.728
Cattle breeder	139	623				
Cattle breeder/finisher	4635	13482	0.91	0.50	1.68	0.770
Cattle finisher	6820	17151	1.02	0.55	1.90	0.954
Farm.Type						0.364
Hill	927	2093				
Low Ground Arable	8131	20176	1.22	0.85	1.75	0.287
Marginal	2499	8892	1.02	0.68	1.52	0.934
Calves						
Calves			1.00	1.00	1.00	0.641
BuyCattle						
No	1392	4981				
Yes	10202	26275	1.40	1.06	1.86	0.017
SheepKept						
No	1943	6007				
Yes	6897	18519	1.08	0.81	1.44	0.603
DucksPres						
No	6336	19404				
Yes	2504	5122	1.10	0.83	1.47	0.510
GeesePres						
No	5474	16334				
Yes	3366	8192	1.00	0.76	1.31	0.986
DeerPres						
No	3095	8434				
Yes	5745	16092	1.36	1.03	1.78	0.027
RabbPres						
No	3095	8434				
Yes	5745	16092	0.94	0.70	1.26	0.695
TimeOnPastureCSH						0.780
0 - 4 months	2423	7003				
5 - 8 months	8133	21522	0.89	0.63	1.26	0.512
9 - 12 months	414	1053	1.02	0.58	1.78	0.943

Table D.1 continued - part 1.*Variables *SilageSupp* and *ConcentratesSupp* refer to the use of Silage and Concentrates supplementation at any time respectively and were derived from Q.29

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
PrevYearCows						
No	5700	13468				
Yes	4347	13335	0.82	0.61	1.11	0.199
PrevYearCalv						
No	5563	12546				
Yes	4484	14257	0.71	0.53	0.94	0.016
PrevYearStHf						
No	662	2411				
Yes	9385	24392	0.98	0.67	1.43	0.908
PrevYearEwes						
No	5682	14868				
Yes	4365	11935	0.99	0.76	1.29	0.944
PrevYearLamb						
No	5017	13834				
Yes	5030	12969	1.17	0.89	1.55	0.257
ThisYearSheep						
No	1860	4750				
Yes	5878	15386	0.99	0.75	1.32	0.964
ThisYearHorse						
No	7224	19029				
Yes	514	1107	1.13	0.70	1.82	0.628
ThisYearCalve						
No	4552	10566				
Yes	3186	9570	0.82	0.62	1.08	0.165
SeparateGrazing						
No	5263	12454				
Yes	4328	12300	1.08	0.82	1.43	0.581
RotationalGrazing						
No	4524	12013				
Yes	5067	12741	0.87	0.66	1.16	0.347
Cograzingsheep						
No	7296	18309				
Yes	2295	6445	0.94	0.71	1.26	0.681
Cograzingoth						
No	9547	24624				
Yes	44	130	1.13	0.46	2.75	0.789
Hay_Spring						
No	8332	23828				
Yes	1361	2879	0.93	0.65	1.34	0.710
Hay_Autumn						
No	8716	24230				
Yes	977	2477	0.84	0.54	1.30	0.435
Hay_Winter						
No	7464	22721				
Yes	2229	3986	1.35	0.98	1.85	0.066

Table D.1 continued - part 2.

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
Straw_Spring						
No	6055	14006				
Yes	3638	12701	0.73	0.56	0.94	0.015
Straw_Summer						
No	7608	20660				
Yes	2085	6047	0.99	0.65	1.52	0.968
Straw_Autumn						
No	5166	12181				
Yes	4527	14526	0.90	0.69	1.17	0.438
Straw_Winter						
No	3205	7910				
Yes	6488	18797	0.96	0.71	1.30	0.810
SilageSupp*						
No	1301	5042				
Yes	8392	21665	1.18	0.80	1.73	0.401
ConcentratesSupp*						
No	848	2356				
Yes	8845	24351	0.93	0.62	1.40	0.742
HayFlooding						
No	10252	27849				
Yes	707	1472	0.99	0.63	1.56	0.979
SilageFlooding						
No	9522	26786				
Yes	1493	2852	1.03	0.73	1.46	0.863
FYM						
No	5192	15426				
Yes	4756	12140	1.04	0.80	1.36	0.760
WaterBodies						
No	5948	16521				
Yes	4173	11840	0.94	0.72	1.22	0.624
FenceOff						
No	6941	18647				
Yes	2799	8780	0.87	0.66	1.15	0.332
DrainTempWaterBod						
No	5095	14909				
Yes	4909	13238	1.17	0.89	1.52	0.257
AccessTempSource						
No	5794	17587				
Yes	4260	10692	1.23	0.95	1.60	0.12
AccessPermSource						
No	6574	18617				
Yes	3548	9750	0.93	0.69	1.25	0.625
FieldMowed2						0.648
No	5193	13888				
Yes less than 50% of fields	2685	7427	0.91	0.67	1.23	0.526
Yes more than 50% of fields	2457	5874	1.05	0.72	1.52	0.807

Table D.1 continued - part 3.

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
FlGrzManag						
No	8124	21864				
Yes	3230	8764	1.02	0.78	1.35	0.863
FlDrugManag						
No	2297	6948				
Yes	9057	23680	1.53	1.10	2.12	0.011
TxDoseEstEach						
No	4971	13810				
Yes	5435	13894	1.14	0.88	1.48	0.332
TxDoseAvHerd						
No	9315	24816				
Yes	1091	2888	0.97	0.59	1.58	0.897
TxDoseHeaviest						
No	9626	24655				
Yes	780	3049	0.81	0.47	1.40	0.460
TxDoseWeigEach						
No	7448	20879				
Yes	2958	6825	1.06	0.79	1.43	0.680
TxTimingFixed						
No	4996	11835				
Yes	4300	12476	0.81	0.57	1.14	0.235
TxTimingWeather						
No	6304	17147				
Yes	2992	7164	1.07	0.78	1.46	0.674
TxTimingLFForecast						
No	7752	21010				
Yes	1544	3301	0.96	0.65	1.43	0.858
SheepTx						
No	1766	4940				
No sheep kept on farm	3878	10590	0.99	0.65	1.49	0.956
Yes	5710	15098	0.98	0.67	1.44	0.926
sex						
female	4007	9565				
male	7516	21433	0.95	0.89	1.02	0.127

Table D.1 continued - part 4.

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
breed						0.023
Aberdeen Angus	1029	2905				
Aberdeen Angus Cross	3325	9284	1.25	1.10	1.43	0.001
British Blue Cross	340	902	1.10	0.89	1.35	0.390
Charolais	97	277	0.97	0.64	1.48	0.894
Charolais Cross	1672	4234	1.17	1.00	1.37	0.051
Holstein Friesian	447	1765	1.25	1.02	1.52	0.032
Limousin	174	380	1.20	0.91	1.58	0.203
Limousin Cross	2291	5362	1.27	1.10	1.47	0.001
Other	1205	3277	1.13	0.96	1.32	0.142
Simmental Cross	1014	2870	1.26	1.07	1.49	0.005
age_days						
age_days			1.00	1.00	1.00	<0.001
season						<0.001
seasonQ1	3853	9942				
seasonQ2	3610	8486	0.99	0.92	1.07	0.810
seasonQ3	2159	7299	0.80	0.74	0.87	<0.001
seasonQ4	1972	5529	0.89	0.82	0.98	0.012
year						
2013	7591	15416				
2014	4003	15840	0.48	0.48	0.49	<0.001
fluke_treated						
No	3723	12723				
Yes	11247	4336	1.09	0.98	1.21	0.116
PH_W_GMEAN_av						
PH_W_GMEAN_av			1.31	1.01	1.70	0.063
MS						0.009
1	285	477				
3	257	800	1.31	0.70	2.46	0.394
4	5862	17536	1.91	1.00	3.62	0.048
5	5182	12431	3.33	0.59	18.79	0.173
0	8	12	2.84	1.19	6.83	0.019
RiverDistance2						
<=500	7883	18181				
>500	3711	13075	0.83	0.64	1.08	0.165

Table D.1 continued - part 5.

Variable	Fluke	No fluke	Odds Ratio	Lower 95% CI	Upper 95% CI	p-value
NDJMeanTempMat						
NDJMeanTempMat			1.07	0.88	1.30	0.481
FMAMeanTempMat						
FMAMeanTempMat			1.45	1.13	1.85	0.003
MJJMeanTempMat						
MJJMeanTempMat			1.69	1.42	2.01	<0.001
NDJMeanRainMat						
NDJMeanRainMat			1.00	1.00	1.01	0.031
ASOMeanTempMat						
ASOMeanTempMat			1.55	1.21	1.97	<0.001
FMAMeanRainMat						
FMAMeanRainMat			1.01	1.00	1.02	0.017
MJJMeanRainMat						
MJJMeanRainMat			1.03	1.02	1.04	<0.001
ASOMeanRainMat						
ASOMeanRainMat			1.01	1.01	1.01	<0.001
Slope						
Slope			2.96	0.03	288.68	0.642
Elevation						
Elevation			1.00	1.00	1.00	0.478

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